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Neuronal activity-dependent protection against apoptotic and oxidative insults

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I hereby declare that the majority of the following work is my own, with the contributions of others indicated where appropriate

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Abstract

Patterns of physiological electrical activity in the central nervous system (CNS) cause long-lasting changes in gene expression that promote neuronal survival. These changes can be mediated by signalling pathways activated by Ca^{2+} influx through synaptic N-methyl D-Aspartate receptors (NMDARs). Identification and study of these, and other neuroprotective signalling pathways of the CNS, is invaluable; as this may one day lead to therapeutic strategies against the deleterious effects of CNS injury or degeneration. The data presented in this thesis focuses on activity-dependent neuroprotection and how it interacts with other signalling pathways to protect against apoptotic and oxidative insults.

A previously unobserved role of activity-dependent neuroprotection in mediating the effects of the neuropeptide PACAP is demonstrated. By promoting cAMP/PKA signalling PACAP triggers neuronal firing activity, which is essential for the neuroprotective effects mediated by PACAP. This firing activity cooperates with direct signalling by PKA in promoting long-lasting CREB-mediated gene expression. The molecular events associated with PACAP mediated stimulation of CRE-dependent gene expression are presented. Investigation of the control of neuronal antioxidant defences by neuronal activity, both on its own and in cooperation with astrocyte-derived support, was also investigated. Neuronal activity is demonstrated to strongly increase the capacity of the antioxidant glutathione (GSH) system, through a program of coordinated transcriptional events. The utilisation, biosynthesis and recycling of GSH is enhanced in neurons, leading to increased resistance against oxidative insults. Since several GSH pathway enzyme genes are regulated by the transcription factor Nrf2, the ability of CDDO-F3, a small molecule activator of Nrf2, to mimic the effect of firing activity on neuronal GSH levels was examined. CDDO-F3 sustains neuronal GSH levels and confers neuroprotection against oxidative insult. These actions are dependent on the presence of astrocytes; whereas Nrf2 mediated regulation of GSH pathway genes is essentially inactive in neurons. Neuronal activity and activation of the astrocytic Nrf2 pathway can cooperate, maintaining neuronal GSH levels and protecting neurons against strong oxidative insults. Collectively this work expands our knowledge as to the molecular mechanisms of activity-dependent neuroprotection, and how such signals may synergise with other protective pathways to promote neuronal health.

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Abbreviations

AC	Adenylate Cyclase
ALS	Amyotrophic Lateral Sclerosis
AMPAR	(2-amino-3-(5-methyl-3oxo-1,2-oxazol-4-yl)propanoic acid) Receptor
AP	Action potential
AP1	Activator protein 1
AraC	Cytocine β-D-arabinofuranoside hydrochloride
ARE	Antioxidant response element
ATP	Adenosine trisphosphate
B/4AP	Bicuculline and 4-aminopyridine
BCNU	Bis-chloroethylnitrosourea
BSO	Buthionine sulfoximine
CamK	Ca^{2+}/Calmodulin-dependent kinase
cAMP	Cyclic adenosine monophosphate
CBP	CREB binding protein
CDDO-F3	2-cyano-3,12-dioxooleana-1,9-dien28-oc acid trifluoroethylamide
CNS	Central nervous system
CREB	cAMP response element binding protein
CRTC1	CREB-regulated transcription coactivator 1
EAAT	Excitatory amino acid transporter
ERK	Extracellular signal-regulated kinase
FOXO	Forkhead box transcription factor
GABA	Gamma-aminobutyric acid receptor
GCL	γGlutamylcysteine ligase

GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	(oxidised) Glutathione disulfide
GSS	Glutathione synthetase
GST	Glutathione S-transferase
GT	γGlutamyl transpeptidase
IsAHP	Apamin insensitive slow after-hyperpolarisation
MAGUK	Membrane associated guanylate kinase
MCB	Monochlorobimane
mGluR	Metabotropic glutamate receptor
MRP1	Multidrug resistance protein 1
NADPH	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor κ light chain enhancer of activated B cells
NMDAR	N-Methyl-D-aspartic acid receptor
NRF2	Nuclear factor (erythroid-derived 2)-like-2
PACAP	Pituitary adenylate cyclase activating peptide
PDZ	PSD95/Disc large/Zona occludens 1
PGC-1α	Peroxisome proliferator-activated receptor γ coactivator 1α
PI3K	Phosphoinositol-3-kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PRX	Peroxiredoxin
PSD	Postsynaptic density

ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBI	Traumatic brain injury
TRX	Thioredoxin
TTX	Tetrodotoxin
xCT	cystine/glutamate transporter
γGluCys	γGlutamylcysteine
ΔΨm	Mitochondrial membrane potential

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Chapter 1
Introduction

1.1 Introduction

Neurons of the central nervous system (CNS) allow their host organism to adapt and survive to their environment. Analogously, neuroprotective pathways exist that allow neurons to adapt and survive against the varied intrinsic and extrinsic stressors of their environment. These pathways are vital, in particular within the adult mammalian nervous system, where regeneration and *de novo* generation of neurons is severely limited: the majority of these neurons must literally last a lifetime. Interest in the neuroprotective pathways of the CNS has piqued due to the rising incidence of neurodegenerative diseases: some of which are considered to occur through an attenuation of intrinsic neuroprotective pathways (Rosen et al., 1993); whilst augmenting the activity of certain pathways has been shown to protect against models of neurodegenerative disease (Reglodi et al., 2004; Tamas et al., 2006a; Vargas et al., 2008; Stack et al., 2010).

Of particular interest to me are the neuroprotective pathways regulated by electrical/synaptic activity, often mediated through signalling by the N-methyl D-aspartate receptor (NMDAR) (Hardingham and Bading, 2010). Signalling via the NMDAR provides a convergence of pathways that allow neurons to adapt to the environment. Specific patterns of NMDAR stimulation can cause changes of synaptic responses, or connectivity (Bliss and Lomo, 1970; Kind and Neumann, 2001); NMDAR activation may also regulate neuroprotective pathways (Papadia et al., 2008; Martel et al., 2009b; Zhang et al., 2009). Neuronal damage may be caused by a variety of stressors, which may be presented in a dynamic fashion throughout the lifespan of an organism; thus it is instructive that a receptor intrinsically linked with the adaptive responses of neurons may also regulate protective responses.

The data presented in this thesis focuses on activity-dependent neuroprotection and how it interacts with other signalling pathways to protect against apoptotic and oxidative insults. This introduction shall attempt to briefly summarise some of the current knowledge of the causes of neuronal damage/death and the protective pathways that attenuate neuronal damage. Thus the topics contained in this

introduction shall include the NMDAR; neuronal death and anti-apoptotic pathways; and oxidative stress and the regulation of antioxidant pathways.

2.1 The NMDA receptor

NMDARs are ionotropic receptors gated by glutamate, the predominant excitatory neurotransmitter in the CNS. NMDARs are present on a number of cell types including astrocytes (Krebs et al., 2003), oligodendrocytes (Kolodziejczyk et al., 2009) and chondrocytes (Ramage et al., 2008). However NMDARs are predominantly expressed in neurons where they mediate glutamatergic neurotransmission in the CNS, in concert with α-amino 3-hydroxy 5-methyl 4-isoxazolepropionic acid receptors (AMPARs) and Kainate receptors. In contrast to other glutamatergic receptors, NMDARs require co-activation with glycine, and are blocked at resting membrane potential by Mg^{2+}, which is released only after neuronal postsynaptic depolarisation; making them both ligand *and* voltage gated, which causes them to act as coincidence detectors of synaptic activity.

Another important aspect of NMDAR function is their permeability to Ca^{2+}. AMPARs and Kainate receptors by contrast predominantly show minimal Ca^{2+} permeability. Ca^{2+} influx through NMDARs causes the recruitment of Ca^{2+} dependent signalling molecules, either directly or through Ca^{2+} binding proteins such as calmodulin (Burgoyne, 2007). Acting as a secondary messenger, Ca^{2+} imparts the potential for NMDARs to elicit long lasting changes to the neuron, in both excitability and gene expression. Differences in the consequences of NMDAR mediated Ca^{2+} influx are dependent on the intensity of NMDAR stimulation, and specifics of the NMDARs activated.

Neuronal NMDARs vary in two important aspects: subunit composition, and cellular location. They are heterotetramers, consisting of two obligatory glycine-binding NR1 subunits, and two NR2 subunits which contain the glutamate binding sites. A less common glycine binding NR3 subunit exists, that can replace one or both NR2 subunits, resulting in a glutamate-insensitive glycine-gated NMDAR (Chatterton et

al., 2002). There are four different NR2 subunits A-D, each differing in their affinity to glutamate, and channel properties (Monyer et al., 1992). The most prevalent in the CNS are NR2A and NR2B (with the exception of cerebellum where NR2C is predominant), existing either separately or as NR1/NR2A/NR2B heterotetramers (Sheng et al., 1994). Their expression is developmentally regulated; in rodents NR2B subunits are highly expressed embryonically, with a subsequent post-natal decline, whilst NR2A subunit expression is severely limited in the first week of life, with expression increasing into adulthood (Monyer et al., 1994). With some notable presynaptic exceptions (Duguid and Smart, 2004), NMDARs are expressed postsynaptically at either synaptic or extra/perisynaptic sites. Extrasynaptic NMDARs are predominantly (but not exclusively) NR2B containing (Tovar and Westbrook, 1999), and are activated by glutamate spillover. Differences in NR2 subunit expression and location influence the long-term consequences of NMDAR activation due to association with different signalling proteins.

Post-synaptic NMDARs are coupled to a large multi-protein signalling complex, known as the post-synaptic density (PSD) due to its appearance under electron microscopy (Sheng and Hoogenraad, 2007). The PSD is made up of various signalling and scaffolding proteins, held together by protein-protein interactions, the most predominant being the PDZ domains (Kim and Sheng, 2004). Through PDZ interactions, the intracellular C-termini of NR2 subunits bind to membrane-associated guanylate kinases (MAGUKs), which in turn bind to scaffolding proteins such as Shank and Homer, and signalling enzymes like neuronal nitric oxide synthase (nNOS), protein phosphatase 2b (calcineurin) and calmodulin-dependent protein kinase II (CaMKII). MAGUKs, through PDZ containing protein stargazin, mediate synaptic targeting of AMPARs (Chen et al., 2000). Thus association with the PSD facilitates NMDAR activation and signal transduction.

The make up of the PSD varies with NMDA subunit composition and location. Dissimilarities between NR2A and NR2B C-termini determine specific signalling molecule interactions. They show greater affinities for different MAGUKs; NR2B for SAP102 and NR2A for PSD-95 (Sans et al., 2000); whilst NR2B subunits

binding to CamKII is stronger than that of NR2A (Barria and Malinow, 2005). Extrasynaptic NR2 subunits associate with MAGUKs and other signalling molecules, however they do not form the dense signalling clusters of the PSD, appreciable by electron microscopy (Petralia et al., 2010).

2.2 Consequences of NMDAR activation

NMDAR mediated Ca^{2+} influx allows the adaptation of the neuron, through changes in local signalling and gene expression, a process known as synaptic plasticity. Specific patterns of NMDAR stimulation may lead to increases (long-term potentiation (Bliss and Lomo, 1970)) or decreases (long-term depression (Mulkey and Malenka, 1992)) in neuronal excitability, and these are thought to underlie a cellular basis of learning and memory (Morris et al., 1986). Similarly, NMDAR mediated changes in local signalling and gene expression can influence the health of the neuron, promoting survival or programmed death pathways. It is beyond the scope of thesis to review the processes behind NMDAR dependent synaptic plasticity. However it is instructive that many of the pathways that underlie synaptic plasticity also play an important role in the death/survival signalling of the NMDAR. Rather than death/survival being the severe conclusion of changes in synaptic plasticity; this convergence illustrates the required dynamic nature of neuronal health signalling. Just as neurons will have to modulate their excitability to adapt to an ever changing environment, so will the signalling that controls their defences and survival. Thus it is important to note that NMDAR signalling can influence neuronal health: its place within a spectrum of physiological states ranging from functional and protected to dysfunctional and vulnerable (Bell and Hardingham, 2011b). Before discussing these NMDAR regulated pathways in greater detail, the mechanisms of cell death shall first be introduced. This is not just to provide context; as certain aspects of cell death are under genetic control, and some neuroprotective pathways act by directly interfering or downregulating cell death signalling.

3.1 Neuronal death and survival

Neuronal death can be characterised through differences in their causes, morphology and the underlying biochemical processes that control them; however due to overlapping phenomenology unequivocal categorisation is fraught with difficulty (Kroemer et al., 2009). The three most distinct categories are autophagy, necrosis and apoptosis, the latter two shall be discussed in greater detail in this introduction. Autophagy is a catabolic process whereby cellular organelles and cytoplasmic material are sequestered in double-membraned vesicles termed autophagosomes, wherein they are degraded by lysosomal enzymes generating ATP (Green et al., 2011). This process allows cells to generate energy when they lack nutrients, or to degrade potentially hazardous internal machinery such as damaged mitochondria (mitophagy). Whilst these processes in general promote cell survival, autophagy sufficient to cause the degredation of the entire cell and its death also occurs; recent evidence suggests that the activation of survival-promoting and death-promoting autophagy are controlled by distinct signalling pathways (McPhee et al., 2010). Also included in this introduction excitotoxicity, an NMDAR dependent cause of cell death that may cause both necrosis and apoptosis, shall be described. Oxidative stress, which may act as both a cause and consequence of neuronal death shall be described in greater detail in a separate section of this introduction. The pathways through which NMDARs signal to promote or impede cell survival shall be discussed. A final sub-section of this chapter shall discuss the role of neurotrophic factors in promoting cell survival; data presented in this thesis describes the role of the neurotrophic factor PACAP in promoting synaptic-activity dependent neuroprotection against apoptotic insults.

3.1a Necrosis

Necrosis is characterised morphologically by swelling of the cell and its organelles, plasma membrane rupture and degeneration, and release of the cytoplasmic contents of the cell (Kerr et al., 1972; Holler et al., 2000; Edinger and Thompson, 2004). In contrast with some other forms of cell death it is not associated with DNA

fragmentation, and is independent of caspases - key cysteinyl aspartate proteases that are essential for apoptosis. Necrotic cell death has long been considered to be an uncontrolled form of cell death, occurring after severe cell trauma. Recent evidence however suggests that necrosis, similar to other forms of cell death, is controlled by a defined signalling pathway regulated by receptor-interacting protein kinases (Holler et al., 2000; Oberst et al., 2011; Zhang et al., 2011a), though this programmed necrosis, or necroptosis, may be a separate modality of cell death in itself (Kroemer et al., 2009). Since necrosis culminates with the release of cellular contents, it may lead to the death of neighbouring cells through release of inflammatory cytokines. Whilst this previously was considered evidence of necrosis as accidental and uncontrolled form of cell death, this release of pro-inflammatory signalling molecules has been shown to play an important role in the defence against viral infection (Edinger and Thompson, 2004). Necrosis occurs during the initial acute phase of an ischemic or excitotoxic episode, as occurs during stroke. Necrosis, as opposed to other forms of cell death, occurs during an ischemic episode when Ca^{2+} entry into the cell through NMDARs is sufficient to cause ionic deregulation of the cell, causing Cl^- and water entry that ruptures the cell membrane (Choi, 1994).

3.1b Apoptosis

Apoptosis is a form of programmed cell death, characterised by cellular and nuclear shrinkage (pyknosis), chromatin condensation and DNA fragmentation, with little or no morphological changes in cytoplasmic organelles (Kerr et al., 1972; Kroemer et al., 2009); in contrast to necrosis these are changes are caused by caspase mediated cleavage and are ATP dependent. Apoptosis of a cell does not allow release of cytoplasmic contents and potential damage of neighbouring cells, and has thus long been considered a controlled form of cell death (Kerr et al., 1972). Apoptosis is required for correct organogenesis in developing embryos, and is essential throughout life to eliminate cells that have suffered DNA damage, to prevent the formation of tumours. Apoptosis plays a key role in the patterning of the CNS, with genetic ablation of key apoptotic regulators such as caspases 3 and 9 and Apaf1 causing ectopic cell masses in forebrain and increased embryonic lethality (Buss et

al., 2006). Apoptosis may also occur in response to neuronal injury and during neurodegeneration, and thus is relevant in both physiology and pathophysiology (Thompson, 1995).

Apoptosis may be activated by two pathways, one extrinsic the other intrinsic. The extrinsic pathway is triggered by activation of dedicated transmembrane death receptors of the tumour necrosis factor superfamily (Ashkenazi and Dixit, 1998). Upon activation these receptors cluster, forming a death-inducing signalling complex at their intracellular domain (Riedl and Salvesen, 2007), that recruits apoptosis-initiator enzymes caspase-8 or 10. This results in the dimerisation of these caspases, which leads to their activation (Boatright et al., 2003). The intrinsic pathway is triggered by the sensing the mitochondrial release of cytochrome-c, an essential component of the electron transport chain where it carries one electron between complexes III and IV, by the cytosolic protein Apaf-1 (Zou et al., 1997). Apaf-1 binds cytochrome-c, and then makes an ATP-dependent conformational change allowing it to form a signalling complex termed the apoptosome, which is made up of seven Apaf-1 molecules arranged in wheel-like structure (Riedl and Salvesen, 2007). This then recruits initiator enzyme caspase-9, which dimerises to its active form (Boatright et al., 2003). The (reversible) activation of initiator caspases of the extrinsic and intrinsic apoptotic pathways, causes the cleavage of apoptosis-executor enzymes caspase-3 and 7, which in turn causes their irreversible activation (Riedl et al., 2001). The cleavage of the substrates of these enzymes lead to the morphological changes associated with apoptosis.

Apoptosis is regulated by the Bcl-2 family of proteins, which can promote or inhibit the initiation of apoptosis. These proteins are characterised by 4 homologous domains (BH1-4) that are intrinsic to their activity. Bcl-2 family proteins can be grouped into three classes: anti-apoptotic members (e.g.: Bcl-2, Bcl-XL), multi-domain pro-apoptotic members (e.g.: BAX, BAK) and pro-apoptotic proteins that are structurally divergent but with a conserved BH3 domain (BAD, BID, BIM and PUMA) (Youle and Strasser, 2008). Once activated BAX or BAK form oligometric structures through BH3 domain binding that permeabilise the outer mitochondrial

membrane, allowing release of cytochrome-c (Tait and Green, 2010). Bcl-2 and Bcl-XL are localised to the mitochondrial membrane, and inhibit the formation of BAX/BAK mediated pores, through binding to BH3 domains. The BH3 only Bcl-2 members inhibit this interaction, disinhibiting BAX and BAK, by binding to the anti-apoptotic Bcl-2 proteins (Youle and Strasser, 2008); and may trigger activation of BAX/BAK (Tait and Green, 2010). Activity of Bcl-2 proteins is regulated by *de novo* gene expression and post-translational signalling cascades. PUMA mRNA expression is upregulated by the tumour suppressor p53 in response to DNA damage (Nakano and Vousden, 2001), whilst BIM expression is regulated by the forkhead box transcription factor-3A (FOXO3a) in response to neurotrophic factor deprivation (Dijkers et al., 2000). BAD is phosphorylated by Akt kinase (Zha et al., 1996) which sequesters it in the cytosol, loss of this signal allows its activation. BIM provides a link between the extrinsic and intrinsic apoptotic pathways, its cleavage by caspase-8 promotes its mitochondrial translocation to inhibit Bcl-2 (Zha et al., 2000). Bcl-2 on the other hand, is regulated at a transcriptional level by CREB (Wilson et al., 1996) and is produced in response to neurotrophic factors (Riccio et al., 1999; Aubert et al., 2006).

Neuroprotective pathways regulated by synaptic activity are able to inhibit cell death by various mechanisms. We recently observed an NMDAR dependent downregulation of PUMA, Apaf1 and caspase-9 mRNA by increased synaptic activity, which conferred protection through inhibition of the intrinsic apoptotic pathway (Leveille et al., 2010). Other mechanisms by which synaptic activity may inhibit cell death shall be discussed in further detail.

3.1c Excitotoxicity

It was first reported over 50 years ago that sustained glutamate exposure caused neuronal death (Lucas and Newhouse, 1957), in a Ca^{2+} dependent process coined excitotoxicity (Olney, 1969; Choi, 1987). During an ischemic episode, as occurs in stroke, a bolus of glutamate is released from dying cells due to reversal of ATP driven glutamate transporters (Rossi et al., 2000), causing excessive activation of

NMDARs and massive Ca^{2+} influx (Tymianski et al., 1993). This creates an ionic deregulation of the cell, involving Cl- and water entry, causing the cell to burst and become necrotic; when cells are exposed to less extreme but still fatal doses of glutamate, Ca^{2+} causes mitochondrial depolarisation and cytochrome c release, which activates the apoptotic programmed cell death pathway of the cell (Choi, 1994, 1996). Excitotoxicity has been shown to mediate neuronal death in traumatic brain injury and epilepsy (Meldrum, 1991; Palmer et al., 1993; Biegon et al., 2004); and has also been implicated in neurodegenerative disorders such as ALS and Huntington's disease (Roy et al., 1998; Zeron et al., 2002).

Due to the extensive evidence of NMDARs role in excitotoxic injury, antagonist based therapies were considered, particularly for treatment of stroke; and showing some efficacy *in vitro* were put into clinical trials (McCulloch, 1992; Lipton and Rosenberg, 1994; Muir and Lees, 1995). Unfortunately, none were successful (Birmingham, 2002; Ikonomidou and Turski, 2002). The failure of these trials could be mitigated by some factors, such as an inability to consistently ensure drug administration during a defined therapeutic window; or the concentrations used, far lower than the equivalent in animal models, due to the psychotomimetic effects of NMDAR antagonists (Birmingham, 2002; Muir, 2006). However this belies the adverse effect of NMDAR blockade; some of these drugs actually reduced survival rate of patients (Davis et al., 2000; Albers et al., 2001). Concurrent with this highly publicised and expensive failure was the emergence of evidence of the neurotoxic effect of NMDAR blockade in animal models (Hwang et al., 1999; Ikonomidou et al., 1999; Pohl et al., 1999; Ikonomidou et al., 2000). This led to the generation of a 'bell-shaped curve' theory of NMDAR mediated toxicity, that both too much and too little NMDAR activation caused neuronal death (Lipton and Nakanishi, 1999). Advances in research of NMDAR dependent signalling has shown this to be an oversimplification; our current understanding of neuroprotective NMDAR signalling and the pathways that mediate its effects shall be introduced.

3.2a Synaptic and Extrasynaptic NMDAR Activity

In the past decades two major additional factors have been determined that influence the consequence of NMDAR activation on neuronal health. A site-specificity of NMDAR Ca^{2+} influx has been established, with synaptic NMDAR activation promoting neuronal health and survival pathways, whilst extrasynaptic NMDAR activation nullify these effects and signal to death-associated pathways (Hardingham and Bading, 2010). A currently contentious issue is a further possible NR2 subunit specificity in determining the consequence of NMDAR activation (Kohr, 2006). Whilst it is intuitive that different signalling pathways would be preferentially associated with the specific C-termini of either NR2, and according examples have been observed associating NR2A with survival-associated pathways and NR2B with death-associated pathways (Liu et al., 2007; Chen et al., 2008; Tu et al., 2010); this subunit specificity has been disputed (von Engelhardt et al., 2007; Martel et al., 2009a). The discrepancy is due to the poor specificity of NR2A-specific antagonists, an often assumed but incomplete segregation of NR2A subunits to synaptic sites and NR2B to extrasynaptic sites (Thomas et al., 2006), and the enigmatic presence of NR2A/NR2B tetraheteromers. A similar controversy surrounds the subunit specificity of synaptic plasticity (Massey et al., 2004; Berberich et al., 2005),

The study of synaptic NMDAR signalling has been greatly advanced by work *in vitro*. In neuronal cultures the $GABA_A$ receptor antagonist bicuculline causes a network disinhibition by releasing the predominantly excitatory neuronal population from tonic inhibition by interneurons (Hardingham et al., 2001b). This treatment, as opposed to bath applied glutamate which would stimulate both synaptic and extrasynaptic NMDARs, has allowed delineation of the pathways triggered by specific increase of synaptic NMDAR activation. Extrasynaptic NMDAR activation can be exclusively studied using the activity-dependent non-competitive NMDAR antagonist MK801, which binds irreversibly to open channel NMDARs (Foster and Wong, 1987; Tovar and Westbrook, 2002). Stimulating with bicuculline and MK801, then washing to remove unbound antagonist, renders an extrasynaptic specificity to subsequent bath applied NMDA stimulation (Bordji et al., 2010; Dick

and Bading, 2010). Using these methods, both post-translational changes in signalling and *de novo* gene expression have been observed, associated with NMDAR dependent signalling to neuronal health.

3.2b The PI3K-Akt kinase signalling cascade

The phosphoinositol-3-kinase (PI3K) and protein kinase B (or Akt kinase) cascade is pleiotropic signalling pathway that in neurons is vital in suppressing the expression of apoptotic genes. PI3K activates Akt kinase indirectly, by mediating the synthesis of phosphatidylinositol trisphosphate which in turn recruits Akt to the plasma membrane; here Akt is activated through phosphorylation by phosphoinositide dependent protein kinase 1 (Brazil et al., 2004). Phosphorylation of targets by activated Akt mediates the neuroprotective signalling by this cascade. Akt activation mediates the phosphorylation and subsequent inactivation of glycogen synthase kinase-3 (Cross et al., 1995), phosphorylation and nuclear exclusion of pro-apoptotic transcription factor FOXO3a (Brunet et al., 1999; Al-Mubarak et al., 2009), and blocks the initiation of apoptosis by tumour suppressor p53 (Yamaguchi et al., 2001). Akt phosphorylates the BH3-only Bcl2 family member BAD, inhibiting its pro-apoptotic activity (Zha et al., 1996). Inhibition of the PI3K/Akt cascade enhances unstimulated neuronal death highlighting its basal requirement in suppressing apoptosis (Papadia et al., 2005)

Synaptic NMDAR stimulation strongly activates PI3K through Ca^{2+} bound calmodulin (Joyal et al., 1997), with a robust anti-apoptotic effect (Papadia et al., 2005; Soriano et al., 2006; Al-Mubarak et al., 2009). Bath application of NMDA by contrast causes a minimal and transient PI3K/Akt activation, dependent on neuronal firing (Soriano et al., 2006; Papadia et al., 2008). Whilst synaptic NMDAR activation causes nuclear exclusion of the pro-apoptotic transcription factor FOXO3a through activation of PI3K (Al-Mubarak et al., 2009), specific extrasynaptic NMDAR stimulation acts in direct opposition, causing active FOXO3a translocation to the nucleus (Dick and Bading, 2010).

3.2c CREB

Another key example of the directly opposing effects of synaptic and extasynaptic NMDAR activation is the cAMP response element binding protein (CREB). CREB is a transcription factor, regulated by synaptic activity in neurons, where it plays an important role in synaptic plasticity and memory (Lonze and Ginty, 2002; Benito and Barco, 2010). It is also vital for survival of central and peripheral neurons (Walton et al., 1999; Lonze and Ginty, 2002; Mantamadiotis et al., 2002), where its activation contributes to the neuroprotective effects of neurotrophins (Bonni et al., 1999; Riccio et al., 1999) and mediates a long-lasting neuroprotection by episodes of synaptic activity that lasts after subsequent suppression of NMDAR activation (Papadia et al., 2005; Zhang et al., 2009). CREB target genes include negative-regulator of apoptosis Bcl2 (Riccio et al., 1999), the neuroprotective peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) (Soriano et al., 2011) and neurotrophic factors BDNF (Shieh et al., 1998; Tao et al., 1998) and PACAP (Fukuchi et al., 2005; Martel et al., 2009b). Some of the data presented in this thesis is a result of investigation of the neuroprotective properties of PACAP; accordingly neurotrophic factors shall be discussed in further detail in this introduction.

CREB activation by synaptic NMDAR stimulation is mediated through the activation of Ca^{2+}/calmodulin kinases including the Ras-ERK 1/2 pathway and CamKIV (Hardingham et al., 2001a; Soriano and Hardingham, 2007). These mediate its phosphorylation at serine-133, allowing its association with CREB binding protein (CBP) a transcriptional co-factor that is necessary for transcription from CRE sites (Chrivia et al., 1993). Extrasynaptic NMDAR stimulation however signals to a dominant CREB shut-off pathway, that supersedes CREB activation by synaptic activity (Hardingham et al., 2002). This is caused by dephosphorylation of ERK 1/2 kinases (Ivanov et al., 2006; Leveille et al., 2008) that limit CREB activation. In addition extrasynaptic NMDAR Ca2+ promotes the nuclear localisation of Jacob which causes the dephosphorylation of CREB (Dieterich et al., 2008).

Another NMDAR/CREB regulated modulator of gene transcription is PGC-1α (St-Pierre et al., 2006; Soriano et al., 2011). A key metabolic regulator (Puigserver and Spiegelman, 2003), its expression in neurons is strongly protective (St-Pierre et al., 2006; Volakakis et al., 2010; Soriano et al., 2011). The loss of PGC-1α activity in Huntington's disease has been demonstrated to be a key aspect of the pathology. Mutant-huntingtin protein, the causative agent of the disease which is characterised by its cellular aggregation (Wanker, 2000), directly interferes with its CREB mediated transcription (Cui et al., 2006) causing neuronal mitochondrial dysfunction. This has recently been shown to be exacerbated by extrasynaptic NMDAR activity, which is increased in Huntington's disease models (Milnerwood et al., 2010), as this impairs PGC-1α expression through synaptic NMDAR signalling (Okamoto et al., 2009). Intriguingly, blockade of extrasynaptic NMDARs by the antagonist memantine (which preferentially blocks NMDA at extrasynaptic sites at lower doses) protected disease model neurons (Okamoto et al., 2009; Milnerwood et al., 2010); thus in this disease, selective blockade of extrasynaptic NMDAR but retention of synaptic NMDAR signalling pathways offers a potentially viable therapeutic strategy.

3.3 Neurotrophic Factors

Neurotrophic factors are signalling molecules released from cells, which through activation of transmembrane receptors promote neuronal survival. Neurotrophic factor signalling plays a vital role in the development and patterning of the CNS; however signalling continues in mature neurons, and has been shown to have potent neuroprotective properties in adult CNS. Two neurotrophic factors that have received considerable attention are brain derived neurotrophic factor (BDNF), and pituitary adenylate cyclase activating peptide (PACAP).

3.3a BDNF

First isolated from pig brain (Barde et al., 1982), BDNF has been the subject of considerable study due to its diverse roles in both developing and mature CNS. It

promotes its effect through activation of two receptors; tyrosine kinase receptor B (TrkB), and the low affinity neurotrophin receptor p75 (Lewin and Barde, 1996). Activation of its receptors by BDNF can activate a variety of internal signal transduction cascades, including the PI3K, Ras-ERK 1/2 and phospholipase C pathways (Huang and Reichardt, 2001). BDNF signalling has been shown to modulate neuronal plasticity, stimulating hippocampal long-term potentiation (Kang and Schuman, 1995; Huang and Reichardt, 2001). BDNF promotes neuronal survival through a number of mechanisms. Through its activation of the PI3K/Akt signalling cascade, it promotes the nuclear exclusion of the pro-apoptotic transcription factor FOXO3a (Zheng et al., 2002). BDNF limits ROS formation caused by excitotoxic NMDA administration *in vitro* by stimulating the activity of the GSH antioxidant pathway, increasing both GR and GPX activity (Mattson et al., 1995). BDNF stimulates the phosphorylation of the neuroprotective transcription factor CREB and subsequent CRE dependent gene transcription by activation of both CaMKIV and Ras-ERK 1/2 (Finkbeiner et al., 1997). Due to its role in CNS patterning, BDNF knockout mice display abnormalities in sensory neuron development and die within the first few weeks of life (Jones et al., 1994; Conover et al., 1995). Genetic ablation of TrkB causes a more severe phenotype, with knockout mice dying shortly after birth (Klein et al., 1993), demonstrating that other neurotrophic factors can activate TrkB. In humans, a polymorphism in the BDNF gene that disrupts its secretion is associated with deficits in declarative memory (Hariri et al., 2003).

Hippocampal BDNF mRNA expression is increased in response to exercise, mediating some of the beneficial effects on the CNS of increased exercise (Cotman and Berchtold, 2002). At a cellular level, BDNF mRNA expression is regulated by CREB (Shieh et al., 1998; Tao et al., 1998), accordingly is upregulated by stimuli that increase the activity of this transcription factor. BDNF expression is upregulated by synaptic NMDAR activity (Hardingham et al., 2002); this constitutes a key mediator of neuroprotection conferred by synaptic activity.

3.3b PACAP

Pituitary adenylate cyclase-activating peptide (PACAP) is a neuropeptide first isolated from the hypothalamus as an activator of cAMP production in pituitary cells (Miyata et al., 1989). It exists in 27 and 38-amino acid forms and binds to three G-protein coupled receptors named PAC1, VPAC1 and VPAC2 which are predominantly coupled to Gαs that promote cyclic adenosine monophosphate (cAMP) production through the activation of adenylate cyclase (AC) (Dickson and Finlayson, 2009). PACAP and its receptors are expressed widely in the CNS, where one of their key functions is neuroprotection. PACAP promotes the protection of cerebellar granule neurons against apoptotic and oxidative insults including ceramide, ethanol and H_2O_2 (Vaudry et al., 2009). PACAP also protects cortical and hippocampal neurons against excitotoxic and apoptotic insults (Shioda et al., 1998; Vaudry et al., 2009). In vivo administration of PACAP reduces neuronal loss and neurological deficits in models of stroke and traumatic brain injury (Reglodi et al., 2002; Chen et al., 2006; Tamas et al., 2006b; Vaudry et al., 2009), excitotoxic striatal lesions (Tamas et al., 2006a) and Parkinson's disease (Reglodi et al., 2004; Reglodi et al., 2006). Given this, PACAP has received considerable attention as a potential therapeutic neuroprotective drug (Somogyvari-Vigh and Reglodi, 2004; Shioda et al., 2006; Brenneman, 2007; Ohtaki et al., 2008; Vaudry et al., 2009).

PACAP promotes neuroprotection by acting directly on neuronal PACAP receptors (Vaudry et al., 2009). The molecular mechanisms that underlie this neuroprotection centre on activation of the cAMP-dependent protein kinase A (PKA), a major effector of intracellular cAMP (Botia et al., 2007; Vaudry et al., 2009). Activation of *de novo* gene expression has been implicated in PACAP-mediated neuroprotection, including c-Fos, BDNF, Bcl-2 and PACAP itself (Frechilla et al., 2001; Falluel-Morel et al., 2004; Fukuchi et al., 2004; Miyashita et al., 2005; Shintani et al., 2005; Aubert et al., 2006; Dejda et al., 2008). Of note, these genes are all regulated by the CREB family of transcription factors. PACAP is known to promote CREB activation under conditions where it is neuroprotective (Racz et al., 2006; Falktoft et

al., 2009), however a causal link between these two phenomena has yet to be determined.

It is generally assumed that PACAP-mediated PKA signalling in neurons triggers neuroprotective gene expression and signal pathways by direct modulation of upstream effectors of these processes. However data presented in this thesis proposes an alternative explanation: that PACAP-induced PKA signalling exerts at least some of its neuroprotective effects indirectly though the enhancement of electrical activity. G-protein coupled receptors that activate cAMP/PKA signals in neurons, such as type I mGluRs and D1-type dopamine receptors, can potentiate synaptic strength, neuronal excitability and ion channel properties (Nguyen and Woo, 2003). PACAP administration has been recently reported to enhance AMPAR currents as well as synaptic NMDAR currents (MacDonald et al., 2007; Costa et al., 2009) and to suppress the apamin-insensitive slow after-hyperpolarization (IsAHP) current (Hu et al., 2010), which can control neuronal excitability. Data presented in this thesis demonstrates an increase in neuronal action potential firing activity, which is required for PACAP mediated neuroprotection: thus PACAP could regulate neuronal health by influencing neuronal excitability.

4 Oxidative stress and antioxidant defences

The oxidative stress theory of ageing (Harman, 1956) proposes that reactive oxygen species (ROS) are the causative agents of the diverse deleterious changes, that over time increase the chance of cell dysfunction and death with age. Whilst this may be an oversimplification and has not been unequivocally proved (Shi et al., 2010), the damage caused by ROS is a hallmark of many neuronal pathologies (Coyle and Puttfarcken, 1993; Dawson and Dawson, 2003; Lin and Beal, 2006; Herring et al., 2010). In healthy cells ROS are neutralised by antioxidant pathways; when this becomes unbalanced in favour of ROS, oxidative stress occurs. Oxidative stress is associated with a number of neurodegenerative diseases such as ALS (Rosen et al., 1993), and neuronal injury such as ischemia (Chan, 2001). Neurons are particularly vulnerable to oxidative stress for several reasons. They show low expression of

antioxidant enzymes, in particular catalase (Mavelli et al., 1982), in comparison to other cell types. Furthermore, certain neuronal signalling pathways are associated with the production of ROS (or reactive nitrogen species), either as a by-product or as the signalling molecule itself. We recently observed the upregulation of genes that promote antioxidant pathways by synaptic activity (Papadia et al., 2008), indicating that the regulation of antioxidants may be a vital protective pathway required for neuronal survival. Data presented in this thesis demonstrates the hitherto unobserved upregulation of the antioxidant glutathione pathway by synaptic activity. The neuroprotective potential of this upregulation, in concert with an alternative drug treatment previously established to regulate the glutathione pathway is also demonstrated. In this chapter, the sources and formation of ROS, the antioxidant pathways that neutralise them, and the transcriptional regulation of antioxidants shall be discussed.

4.1 Reactive oxygen and nitrogen species

Free radicals are any atom, ion or molecule that contains unpaired electron orbitals, that are capable of independent existence (Halliwell, 1992). The majority of free radicals are highly reactive and thus short lived, a notable exception being molecular oxygen O_2; which contains two singlet electrons with the same spin number, which limits its reactivity. Due to this parallel spin, oxygen will preferentially react one electron at a time. Oxygen is of course the electron acceptor required for energy production through aerobic respiration in the mitochondria of all higher organisms. However, whilst oxygen is required for aerobic life, high concentrations of oxygen have long been known to be toxic (Gerschman et al., 1954); with enhanced toxicity closely correlated to increased metabolism.

Within the electron transport chain of mitochondria, cytochrome-c oxidase (complex IV) reduces O_2 to H_2O through the addition of four electrons; this reduction occurs in a step-wise fashion with partially reduced forms of oxygen bound to metal ions in the enzyme. However, premature "leakage" of electrons from complexes I and III react with oxygen to form the radical superoxide $O_2^{\bullet -}$ (Boveris and Chance, 1973; Turrens,

1997; Brookes, 2005). Put simply, the mitochondrial production of superoxide is largely regulated by two factors: the presence of oxygen to act as an electron acceptor, and flow of electrons across the electron transport chain (Turrens, 2003). Thus increased oxygen concentration will increase superoxide formation, as will impediment of electron transfer through the respiratory chain. Transfer of electrons through the electron transport chain is influenced by the mitochondrial membrane potential ($\Delta\Psi m$), as this determines the protonmotive force that drives ATP synthase. Elevated aerobic respiration increases $\Delta\Psi m$, which can lead to an insufficiency of the substrate ADP, causing reduction of the respiratory chain, allowing the monovalent reaction of electrons with O_2 (Turrens, 2003). Damage of members of the electron transport chain by ROS or reactive nitrogen species also increases $O_2^{\bullet -}$ formation (Bolanos et al., 1995; Duchen, 2000). Reduction of oxygen concentration reduces mitochondrial respiration through inactivation of complex I; however subsequent reperfusion of oxygen causes $O_2^{\bullet -}$ production until this inactivation can be reversed (Galkin et al., 2009).

Mitochondria import cytoplasmic Ca^{2+}, which increases aerobic respiration and ATP synthesis (Jouaville et al., 1999; Duchen, 2000; Duchen et al., 2008). Mitochondrial Ca^{2+} uptake initially reduces $\Delta\Psi m$ (Garcia et al., 2005), with the increase of mitochondrial respiration a compensatory mechanism. Ca^{2+} entry through NMDARs increases mitochondrial respiration, stimulating $O_2^{\bullet -}$ formation (Dugan et al., 1995; Reynolds and Hastings, 1995; Garcia et al., 2005). Superoxide is also generated in response to extrinsic stimuli such as xenobiotics (Cohen and d'Arcy Doherty, 1987; Savolainen et al., 1998) ultra-violet radiation (Devary et al., 1992); and enzymatically by a number of enzymes. One of the most relevant to neurons is NADPH oxidase, which has been shown to be the primary source of $O_2^{\bullet -}$ formation after NMDAR stimulation (Brennan et al., 2009); an observation that is testament to the mitochondrial homeostatic mechanisms that limit ROS formation.

Whilst $O_2^{\bullet -}$ is not itself the most reactive of ROS, it is membrane permeable and thus able to cause widespread oxidative damage throughout the cell. $O_2^{\bullet -}$ is quickly dismutated to H_2O_2 by superoxide dismutase enzymes (SOD) (Weisiger and

Fridovich, 1973). Mutations in the cytoplasmic Cu/Zn SOD1 gene are associated with ALS (Rosen et al., 1993), and mitochondrial Mn SOD2 knockout mice exhibit marked mitochondrial damage and neurodegeneration and survive for only three weeks (Lebovitz et al., 1996). H_2O_2 is also produced in neurons by monoamine oxidase, as a by-product of the catabolism of dopamine (Halliwell, 1992; Coyle and Puttfarcken, 1993). Recent evidence suggests that H_2O_2, produced indirectly through activation of NADPH oxidase and subsequent $O_2{\cdot}^-$ dismutation, may act as a secondary messenger in neurons (Avshalumov et al., 2005; Forman et al., 2010; MacFarlane et al., 2011). Whilst it is itself toxic at high concentrations, H_2O_2 can react with Fe^{2+} ions to form the more dangerous hydroxyl radical (·OH) by the Fenton reaction (Halliwell, 1992):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {\cdot}OH + OH^-$$

Hydroxyl radicals are extremely reactive, and quickly react with membrane lipids, carbohydrates and DNA. ·OH is able to abstract a hydrogen atom from the DNA deoxyribose phosphate backbone, causing strand cleavage (Hertzberg and Dervan, 1984). Abstraction of hydrogen atoms from lipid side chains by ·OH produces a carbon-centred lipid radical, which through reaction with oxygen and neighbouring lipids sets of a free radical chain reaction, both self propagating and producing more lipid hydroperoxides (Halliwell, 1992). ·OH can also react with neurotransmitter dopamine, producing the toxin 6-hydroxydopamine (Slivka and Cohen, 1985). If oxidative damage is sufficient, apoptotic pathways are activated, destroying the cell. In contrast with other ROS, ·OH do not have a dedicated enzymatic antioxidant pathway that specifically neutralises them, though evidence suggests that they can be neutralised by broad-specificity antioxidants such as ascorbate and thioredoxins (Cohen, 1994; Das and Das, 2000). This is perhaps indicative of the transient lifespan of ·OH radicals, and their quick reaction with substrates; and accordingly the importance of neutralising H_2O_2 before it can form ·OH radicals.

Reactive nitrogen species may also have a cytotoxic effect. Nitric oxide (NO), is produced by nitric oxide synthase in response to NMDAR activation, and modulates

neuronal excitability through nitrosylation of free cysteine residues of ion channel and neurotransmitter receptors, and through stimulating production of the secondary messenger cGMP by the enzyme guanylate cyclase (Baranano et al., 2001; Ahern et al., 2002). Reaction of NO with $O_2\bullet^-$ produces the anion peroxynitrite ($ONOO^-$), which is itself reactive and toxic but may also degrade to ·OH (Halliwell, 1992). Both NO and $ONOO^-$ can cause inhibition of the mitochondrial electron transport chain, that may lead to cell death (Heales et al., 1999); though the toxic effect of NO overproduction (but not $ONOO^-$) can be largely attenuated by $O_2\bullet^-$ scavenging by superoxide dismutases (Lipton et al., 1993), indicating that $ONOO^-$ is the more potent toxin.

4.2 Antioxidant Pathways

Oxidative stress occurs when there is an imbalance between the production of ROS and the cell's capacity to deal with them through antioxidant systems. There are a number of heterogeneous antioxidant molecules and associated enzymes within the CNS; whilst some share substrate specificity, specific localisation largely limits the redundancy between pathways. Antioxidant molecules/enzymes in the CNS include (but are not limited to): glutathione (GSH), thioredoxin, peroxiredoxin, and ascorbate.

4.2a Glutathione

GSH (Hopkins, 1921) is a tripeptide of atypical linkage, consisting of glutamate, glycine and cysteine; containing an isopeptide bond between the γ carboxy side chain of glutamate and the amine group of cysteine, with a regular peptide link between cysteine and glycine. It is the most abundant thiol containing compound in mammalian cells (Meister and Anderson, 1983). GSH is the electron donor in the reduction of peroxides to water catalysed by the enzyme glutathione peroxidase (GPX) (Mills, 1957). GPXs are homotetrameric enzymes, with the exception of GPX4 which is a monomeric enzyme that is able to reduce lipid hydroperoxides (Lu and Holmgren, 2009). So far 8 GPXs have been identified through sequence

homology (though some do not contain the vital selenocysteine active site and consequently do not show GPX activity (Nguyen et al., 2011)). The five selenocysteine containing GPXs are segregated in their localisation at either whole body or sub-cellular level: GPX1, the most prevalent, is ubiquitously found in cytosol, GPX2 is largely gastrointestinal specific, GPX3 is extracellular and concentrated in plasma, GPX6 is mainly localised to the olfactory bulb (Brigelius-Flohe, 1999; Lu and Holmgren, 2009). GPX4 is localised to lipid membranes, where it maintains membrane integrity; its importance is highlighted by the embryonic lethality of GPX4 knockout, in comparison to other GPX knockouts which are viable despite a sensitisation to oxidative stress (Brigelius-Flohe, 2006). Reduction of H_2O_2 or lipid hydroperoxides by GPXs can be expressed as the following formula:

$ROOH + 2\ GSH \rightarrow ROH + H_2O + GSSG$

Reduction of peroxides by GPX is a three step process: the selenocysteine active site is oxidised to selenic acid by peroxide, this then reacts with GSH releasing H_2O, this GSH-GPX intermediate is then reduced by GSH back to the slenoate anion form producing glutathione disulfide (GSSG). This two step process impedes the stabilisation of potentially damaging thiyl radicals from GSH, which may occur in highly oxidising conditions (Cohen, 1994). GSSG is then recycled back to GSH by glutathione reductase (GR) (Conn and Vennesland, 1951; Mapson and Goddard, 1951), which transfers an electron from reduced nicotinamide adenine dinucleotide phosphate (NADPH). Whilst there is both separation and overlap in the various peroxide degrading pathways, the GPX pathway is thought to be the most vital in neurons, causing the greatest damage when it is inhibited (Mitozo et al., 2011).

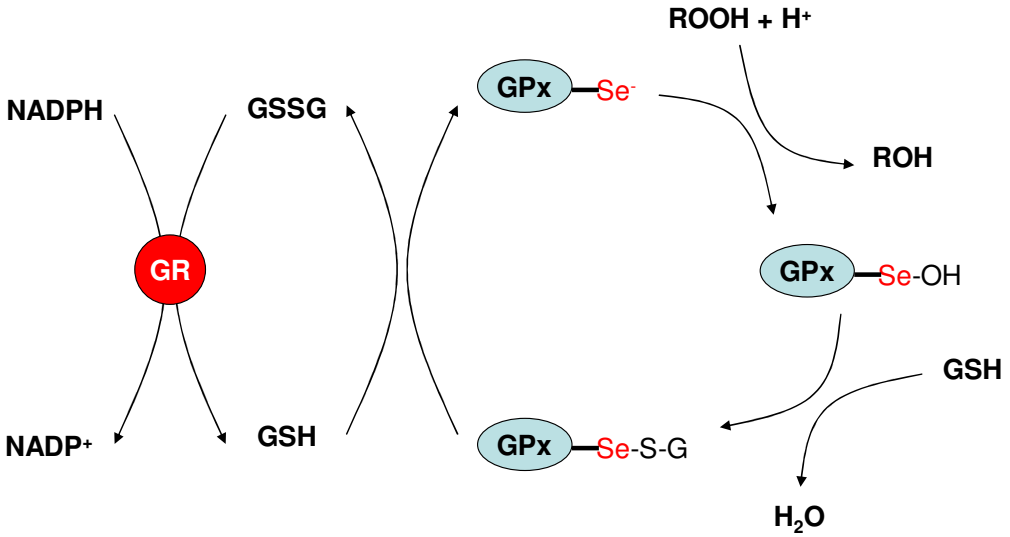


Figure I.1. Catalytic Redox Cycle of GSH. Selenocysteine in the glutathione peroxidase (GPX) active site is oxidised to selenic acid by H_2O_2 or lipid hydroperoxides, then reduced by GSH in a two-step process back to the selenolate anion form. GSSG is then reduced to two molecules of GSH by the NADPH consuming glutathione reductase (GR) enzyme (adapted from (Lu and Holmgren, 2009)).

GSH may also react non-enzymatically with oxidised thiols, forming a disulfide bond. These are reduced by the glutaredoxin (GRX) family of enzymes. In mammalian cells two GRXs have been identified, cytosolic GRX1 and mitochondrial GRX2 (Gladyshev et al., 2001). These enzymes maintain the thiol redox status of proteins within the cell. They contain two cysteines within their active site that act as reducing equivalents, forming a disulfide bond. In a manner similar to GPX, this is subsequently reduced in a two step process by GSH, producing GSSG, which is subsequently reduced by GR (Holmgren, 1989; Beer et al., 2004). GRX2 maintains the complex I redox status within the electron transport chain, preserving mitochondrial function during oxidative stress (Karunakaran et al., 2007).

GSH is also the substrate of glutathione S-transferase (GST) (Booth et al., 1961), which forms GSH-conjugates to proteins through Michael-addition, which are then excreted from the cell by transmembrane multidrug resistance proteins such as MRP1 (Cole and Deeley, 2006). This pathway is key in defending the cell from

xenobiotic compounds (Hayes et al., 2005). GSH may also act as a cellular reservoir for cysteine, which would otherwise auto-oxidise to cystine (Cotgreave and Gerdes, 1998).

Under normal conditions GSH is found in it reduced form, due to the activity of GR (Meister and Anderson, 1983); however after periods of oxidative stress, which significantly reduce the GSH:GSSG ratio, then *de novo* GSH synthesis is required. Glutathione synthesis requires the action of two ATP-dependent enzymes γ glutamyl-cysteine ligase (GCL) and glutathione synthetase (GSS). GCL is a heterodimer consisting of a catalytic (GCLC) and modulatory (GCLM) subunit. GCLC is the larger subunit, containing the active site responsible for the ATP-dependent bond formation between cysteine and the γ-carboxyl group of glutamate. This ligation is the rate-limiting step of GSH synthesis, and levels of GCLC and bioavailability of cysteine are considered to be the two key determinant factors influencing cellular GSH content (Griffith, 1999); accordingly GCLC knockout in mice causes embryonic lethality (Dalton et al., 2000). GCLM is smaller, and has no catalytic properties on its own, but serves to increase activity of GCL by decreasing *Km* for glutamate and ATP (Yang et al., 2007); though GCLC may catalyse γ-glutamylcysteine (γGC) synthesis in the absence of GCLM. The activity of GCL may also be modulated by post-translational modification; phosphorylation of the active site of GCLC by PKA, CAMKII and protein kinase C lead to a decrease in GCL activity (Sun et al., 1996); further evidence suggests modulation by myristoylation and oxidation (Franklin et al., 2009). Another important modulation of GCL activity is non-allosteric inhibition of the catalytic site by GSH itself (Richman and Meister, 1975), thus providing a direct negative feedback of GSH synthesis. GCLM reduces the K_i of GCL by GSH (Huang et al., 1993), thus increasing the activity of the enzyme. GSS binds glycine to the cysteine moiety of γGC; this enzyme is not as well studied as GCL, though its deficiency in humans caused by a very rare autosomal recessive mutation has been described (Njalsson, 2005).

Within the CNS, GSH and the enzymes responsible for its synthesis are found in greater concentration in astrocytes than neurons (Raps et al., 1989). Synthesis of GSH in astrocytes has been shown to protect neurons from oxidative insult (Dringen et al., 1999; Shih et al., 2003), and co-culturing of astrocytes with neurons increases neuronal GSH levels (Diaz-Hernandez et al., 2005). Astrocyte GSH synthesis is thought to "feed" neuronal GSH levels, exporting GSH via the transporter MRP1 (Minich et al., 2006). Neurons do not express GSH uptake transporters (Vargas and Johnson, 2009), thus it is believed that GSH exported from astrocytes is subsequently hydrolysed to its precursors in the extracellular space; first to cysteine-glycine by γglutamyl-transpeptidase (Dringen et al., 1997a), which is then split by aminopeptidase N (Dringen et al., 2001). Cysteine, which is considered to be the limiting substrate in GSH synthesis (Vargas and Johnson, 2009), is imported by excitatory amino acid transporters (EAATs); genetic ablation of EAAT3 causes a reduction of neuronal GSH levels (Aoyama et al., 2006). Cysteine is readily oxidised to cystine in the extracellular space, and neurons, in contrast to astrocytes, do not express the cystine/glutamate exchange transporter XCT (Sato et al., 2002), and cannot utilise cystine for GSH synthesis. Thus extracellular hydrolysis of GSH to cysteine may allow for more efficient neuronal cysteine uptake.

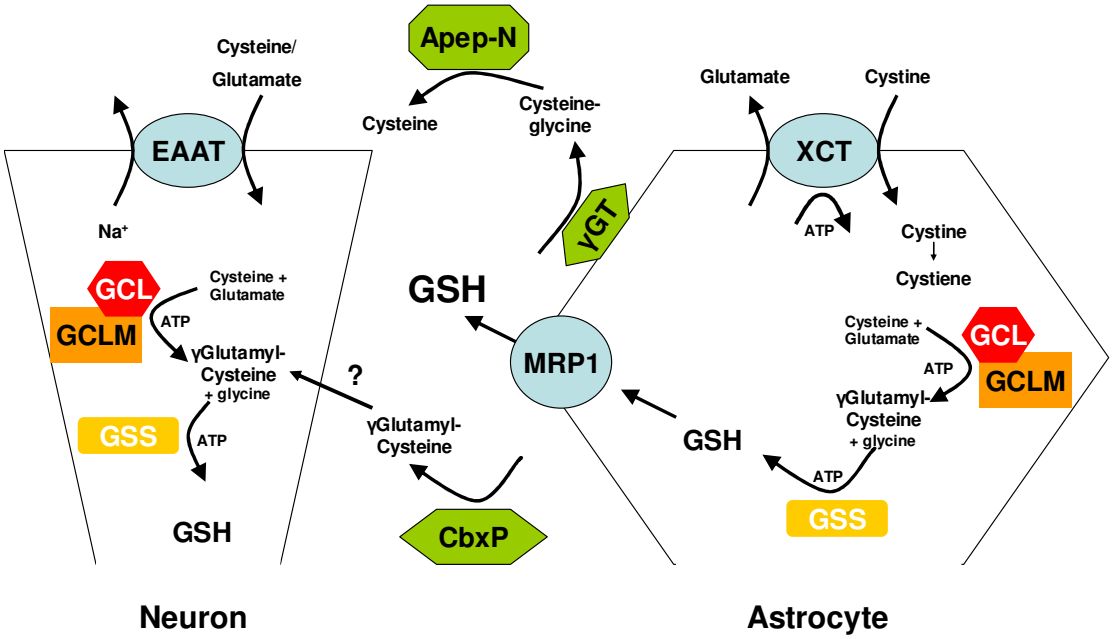


Figure I.2. GSH Synthesis in Neurons and Astrocytes. γGlutamyl-cysteine ligase (GCL) catalyses the binding of γ-carboxyl chain of glutamate and the N-terminus of cysteine, γglutamylcysteine is bound to glycine by GSH synthetase (GSS), forming GSH. GSH synthesis is greater in astrocytes than neurons. GSH is exported from astrocytes by transporter MRP1. Extracellular GSH is hydrolysed, first by γGlutamyl transpeptidase (γGT) to cysteine-glycine, then Aminopeptidase N (Apep-N), releasing cysteine. Neurons import cysteine by excitatory amino acid transporters (EAAT). Astrocytes express glutamate/cystine antiporter XCT, allowing them to import cystine for utilisation in GSH synthesis. Data presented in this thesis suggest that γglutamylcysteine may be taken up by neurons; GSH could be hydrolysed by carboxypeptidases (CbxP) to produce this dipeptide, however a neuronal γglutamylcysteine transporter has not yet been identified (adapted from (Shih et al., 2003)).

Pharmacological depletion of GSH by the potent GCL inhibitor buthionine sulfoximine (Griffith and Meister, 1979) causes neuronal mitochondrial damage (Jain et al., 1991), and a sensitisation to oxidant damage (Mizui et al., 1992; Wullner et al., 1996). Moreover GSH depletion has been implicated in several neurodegenerative diseases, such as ALS (Chi et al., 2007; Babu et al., 2008), Alzheimer's disease (Liu et al., 2004) and Parkinson's disease (Perry et al., 1982), where its loss from the substantia nigra is pre-symptomatic and one of the first biochemical markers of the pathology. Thus the importance of GSH levels in neurons is well documented, and therapeutic strategies have been developed that aim to maintain GSH levels in neurons, through supplementation with membrane-permeable ethyl-ester forms of GSH or γGluCys (Drake et al., 2002), or through upregulation of GCL subunit mRNA expression (Vargas et al., 2008).

4.2b Ascorbic acid

The human necessity for ascorbate (vitamin C) is commonly associated with its role in the stimulation of collagen synthesis, with deficiency of dietary ascorbate causing scurvy (Peterkofsky, 1991). However in the body ascorbate is most concentrated in the CNS (Hornig, 1975), where it acts as a neuromodulator, enzyme co-factor and antioxidant. Ascorbate acts as a broad-spectrum free radical scavenger, neutralising both ROS and RNS to form its oxidation products semi-dehydroascorbate (ascorbyl radical – one electron oxidation) and dehydroascorbate (two electron oxidation) (Rice, 2000). Oxidised ascorbate is either degraded, or reduced (and thus recycled) by GSH utilising enzymes GRX and dehydroascorbate reductase (Meister, 1994; Fornai et al., 1999). Intravenous injection of either ascorbate or dehydroascorbate produce similar increases in reduced ascorbate levels in the CNS, whilst CNS ascorbate levels are maintained during limiting conditions to a greater extent in comparison to other organs, suggesting that ascorbate is actively maintained by GSH dependent recycling in the CNS (Hornig, 1975; Rice, 2000). The broad substrate specificity of ascorbate means it can neutralise ·OH and $ONOO^-$ radicals, which in contrast to $O_2{\cdot}^-$ or H_2O_2 are not neutralised by a specific enzymatic antioxidant

pathway (Cohen, 1994). Ascorbate can compensate for loss of GSH in cells, and vice versa: administration of membrane permeable GSH ethyl ester prevents the onset of scurvy in ascorbate deficient guinea pigs (which unlike rats/mice and similar to humans cannot synthesise ascorbate); whilst ascorbate administration prevents tissue damage caused by BSO treatment (GCL inhibition) (Meister, 1994). Within cells of the CNS, ascorbate is found in greater concentrations in neurons than astrocytes, a reversal of the observed localisation of GSH (Rice, 2000). The GSH dependence of ascorbate recycling further highlights the importance of the GSH antioxidant pathway in neutralising ROS in the CNS.

4.2c Thioredoxins and peroxiredoxins

The thioredoxin (Trx) family of enzymes are thiol based reducing enzymes, typified by a conserved active site (Cys-Gly-Pro-Cys). The active site of Trx reduces disulfide binds of proteins that occur due to oxidative stress, accepting an electron which in turn causes the formation of a disulfide bond between the cysteines of the active site (Holmgren, 1989). This inactivates the enzyme; subsequent reactivation occurs through disulfide bond reduction by Trx reductases (TrxR), NADPH utilising selenocysteine enzymes that are the only enzymes capable of reducing, and thus recycling, the active site of Trx (Lu and Holmgren, 2009). In the mammalian CNS, Trxs and TrxRs are segregated sub-cellularly: Trx1 and TrxR1 are located in the cytosol, whilst Trx2 and TrxR2 are located in the mitochondria (Patenaude et al., 2005). Trxs have diverse roles in the cell and are vital for cell function; genetic ablation of either Trx1 or Trx2 cause embryonic lethality (Matsui et al., 1996; Nonn et al., 2003). Through their reduction of disulfide bonds they can regulate the thiol redox status of many target proteins to maintain their function (Arner and Holmgren, 2000). Trxs can modulate the activity of transcription factors that regulate the viability of the cell: Trx activity causes the activation of transcription factor AP1 (Hirota et al., 1997), whilst downregulating activity of NFκB (Hirota et al., 1999). Trxs also act as electron donating co-factors for ribonucelotide reductase, which is required to provide deoxyribonucleotides for DNA synthesis (Holmgren, 1989; Arner and Holmgren, 2000). Whilst GSH and Grx can also reduce disulphide bonds

of proteins and thus compensate for some of the reactions mediated by Trxs, the rates observed by Trxs are orders of magnitude faster, indicating these two enzymatic pathways fulfil distinct roles in the cell (Holmgren, 1989; Arner and Holmgren, 2000). In the mitochondria Trx2 interacts with members of the electron transport chain, its overexpression increases mitochondrial membrane potential ($\Delta\Psi$) (Damdimopoulos et al., 2002).

The antioxidant properties of Trxs are important for cell viability, and their overexpression decrease ROS in cells, whilst conversely their inhibition increase oxidative damage (Yoshida et al., 2005). Trx overexpressing mice display a reduction in oxidative damage following ischemia, and are vital for neuronal protection against oxidative stress (Patenaude et al., 2005; Bell and Hardingham, 2011a). The antioxidant effects of Trxs, beyond regulation of protein function and transcriptional activity, can be mediated by their action as free radical scavengers (Das and Das, 2000), and their regulation of the peroxide reducing enzymes peroxiredoxins (Prx) (Bell and Hardingham, 2011a). So far six mammalian Prxs have been identified, Prxs1-5 contain a two cysteine catalytic site, whilst Prx6 has only a single cysteine (and is not regulated by Trx) (Patenaude et al., 2005; Bell and Hardingham, 2011a). The 2-cysteines of the Prx catalytic site perform specific roles in H_2O_2 reduction: one cysteine performs the peroxidatic reaction with H_2O_2, oxidising the thiolate group to sulfenic acid (-SOH). The other "resolving" cysteine reacts with the sulfenic acid group, forming a disulfide bond and releasing H_2O (Bell and Hardingham, 2011a). This disulfide bond is then reduced by Trx, restoring Prx activity. Under oxidising conditions the Prx-SOH can be further oxidised to sulfinic ($-SO_2H$) and even sulfonic ($-SO_3H$) acid (Chang et al., 2004); since hyperoxidised peroxidatic cysteine is not a substrate for the resovling cysteine of Prx, this effectively inactivates the enzyme. This is thought to occur during normal regulation of oxidative stress (rather than solely under extremely oxidising conditions (Lim et al., 2008)). The reactivation of Prxs by Trx is slower than the peroxide reducing rate of Prx: direct measurement of Prx activity reveals a 100 fold greater peroxidatic activity than indirect measurement of activity by TrxR NADPH consumption (Peskin et al., 2007). Prx-SO2H is reactivated in an ATP dependent manner by the enzyme

sulfiredoxin (Chang et al., 2004), which catalyses the formation of a sulfinic acid phosphoric ester on Prx, which can be subsequently reduced by Trx (Rhee et al., 2007). Prx-SO3H does seem to cause an irreversible inhibition of Prx activity (Lim et al., 2008); however reversible cysteine hyperoxidation may allow burst activity of Prx, neutralising sudden increases in H_2O_2 without being limited by Trx or TrxR activity.

Whilst both pathways share a diversity in substrate specificity and sub-cellular localisation, the causes underlying the overlapping role of peroxide neutralisation by GPXs and Prxs is likely to extend further than a convergent evolution of both systems (Flohe et al., 2011), or the restricted bioavailability of selenium for GPX expression (Lu and Holmgren, 2009). Inactivation of GPXs in oxidising conditions appear slower than that observed of Prxs; whilst Prxs have a greater ability in neutralising RNS (Flohe et al., 2011). Furthermore, genetic regulation in response to external stimuli could be different. We recently observed upregulation of sulfiredoxin mRNA in response to synaptic activity; this was coordinated with downregulation of *Txnip*, a FOXO regulated protein that binds Trx and inhibits Trx activity (Papadia et al., 2008). Sulfiredoxin is also upregulated by the ROS-responsive transcription factor Nrf2 in response to oxidative stress or activators of Nrf2 (Soriano et al., 2009). Research presented in this thesis demonstrates that genes encoding enzymes of the GSH pathway are similarly upregulated by synaptic activity and by activation of the Nrf2 transcription factor; however these increases are by independent mechanisms.

4.3 Transcriptional control of antioxidant pathways

Whilst the production of ROS occurs as a by-product of aerobic respiration, their production may be increased by a number of factors, including increased cellular metabolism, xenobiotic compounds and ultra-violet radiation (Cohen and d'Arcy Doherty, 1987; Devary et al., 1992; Dugan et al., 1995; Savolainen et al., 1998; Garcia et al., 2005). Thus antioxidant responses must be inherently dynamic. Whilst antioxidant pathway activity can be regulated by post-transcriptional changes

(Franklin et al., 2009), changes in gene transcription also play a vital role in regulating the cellular response to oxidative stress. A key transcription factor in regulating these responses is the nuclear factor (erythroid-derived 2)-like-2 (Nrf2).

4.3a Nrf2

Nrf2 is a redox sensitive transcription factor belonging to the cap 'n' collar subfamily of basic region leucine zipper transcription factors (Zhang, 2006) that binds to the antioxidant responsive element (ARE) (Nguyen et al., 2000), leading to the transcription of phase II detoxifying and antioxidant enzymes (Vargas and Johnson, 2009). Its redox sensitivity is believed to be mediated by its interaction with its negative regulator Keap1, a cytoplasmic actin-bound cysteine-rich protein, which in non-stressed conditions tightly binds Nrf2 to its C-terminal Kelch domain. This sequesters Nrf2 in the cytoplasm, and actively promotes its ubiquitination by E3 ubiquitin ligase and subsequent degradation in the proteasome (Zhang et al., 2004). Under oxidative conditions Nrf2 is released from Keap1, a process that seems dependent on redox-sensitive cysteine residues in the linker region of the Keap1 protein (Zhang, 2006). In addition, phosphorylation of Nrf2 by protein kinase C (PKC) and PI3K enhance Nrf2-mediated transcription from the ARE by disruption of the interaction between Keap1 (Huang et al., 2002; Nakaso et al., 2003).

The phase II detoxifying genes promoted by Nrf2 activation of the ARE consist of a battery of genes that prevent or reduce cellular damage in a number of tissues, including the CNS. Examples include the peroxiredoxin reducing enzyme sulfiredoxin (Soriano et al., 2009), and haemoxygenase 1 (Vargas et al., 2005) which breaks down pro-oxidant haem group to radical scavenging bile pigments biliverdin and bilirubin. ARE dependent transcription also upregulates a cascade of enzymes intrinsically involved with the GSH pathway. GCLC, GCLM, GSS, GR, GPX2 and several GSTs all contain an ARE within their promoters and are upregulated by Nrf2 activation (Vargas and Johnson, 2009). Another Nrf2-mediated gene is the GSH exporter MRP1 (Hayashi et al., 2003) which mediates the supply of neurons with

GSH precursors. Thus Nrf2 activation in astrocytes, leads to a positive antioxidant protection in neurons (Shih et al., 2003; Vargas et al., 2008; Calkins et al., 2010). Recently naturally occurring compounds have been shown to disrupt the Nrf2-Keap1 interaction; isothiocyanates such as sulforaphane (McMahon et al., 2001) isolated from broccoli, and triterpenoids such as oleanolic acid (Dinkova-Kostova et al., 2005) isolated from garlic, potently increase phase II antioxidant gene expression. Synthetic triterpenoids have been produced that exhibit greater potency as protective compounds (Dinkova-Kostova et al., 2005). These compounds cause disinhibition of Nrf2 which confers their powerful anti-carcinogenic and cytoprotective effect. One synthetic triterpenoid in particular, 2-cyano-3,12-dioxooleana-1,9-dien28-oic acid-trifluoroethylamide (CDDO-F3), has been shown to strongly upregulate haemoxygenase 1 expression and confer neuroprotection in mouse models of ALS and Huntington's disease (Pitha-Rowe et al., 2009; Stack et al., 2010; Neymotin et al., 2011). In data presented in this thesis, we utilised this drug to investigate the effects of stimulating the Nrf2 pathway in neurons and astrocytes.

The Nrf2 transcriptional pathway seems stronger in astrocytes than neurons (Kraft et al., 2004; Vargas et al., 2008; Chen et al., 2009; Vargas and Johnson, 2009), however there are reports of Nrf2 mediated transcription in neurons (Escartin et al., 2011; Hota et al., 2011). Whilst we have observed the neuroprotective effects of neuron specific overexpression of Nrf2 (Soriano et al., 2008b), data presented in this thesis raises doubt over the activity of Nrf2 specifically in neurons, with the canonical Nrf2 genes GCLC and GCLM unaffected by pharmacological or genetic manipulation in neurons. This apparent contradiction with previously published results, which include our own, may be due to the highly potent antioxidant and neuroprotective effect of stimulating the Nrf2 pathway in astrocytes. Genetic regulation of antioxidant pathways by NMDAR mediated signalling is likely through alternative transcription factors.

4.3b NMDAR regulated transcription: AP-1 and FOXO

As mentioned previously, we recently observed the downregulation of the Trx inhibitor Txnip by synaptic NMDAR activity, through nuclear exclusion of FOXO (Papadia et al., 2008). We also observed the upregulation by synaptic NMDAR activation of the neuroprotective pro-antioxidant gene sulfiredoxin by the pleiotropic transcription factor AP-1. AP-1 is formed from a noncovalent dimerisation by members of the Fos and Jun family of proteins in response to a variety of external stimuli (Angel and Karin, 1991). Expression of Fos and Jun family member mRNAs are quickly elevated by NMDA receptor activation (Morgan and Curran, 1988; Condorelli et al., 1994). NMDAR activation also mediates phosphorylation dependent stabilisation of dimerised AP-1, facilitating its transcriptional activity (Schwarzschild et al., 1997). Whilst it is unclear if there is a synaptic/extrasynaptic bias in the NMDAR dependent activation of AP-1 mediated transcription; bath applied glutamate is sufficient to drive AP-1 activation (Condorelli et al., 1994; Schwarzschild et al., 1997). However cFos is a CREB regulated gene (Sheng et al., 1990), and we observed no induction of sulfiredoxin mRNA by extrasynaptic NMDAR stimulation (Papadia et al., 2008). We have recently identified AP-1 sites in the promoters of a number of pro-antioxidant genes (Soriano et al., 2009), which suggests that synaptic activity could mediate neuroprotection against oxidative insult through the increased expression of AP-1 regulated genes, alongside concurrent suppression of FOXO regulated genes.

5 Neuronal activity dependent protection against apoptotic/oxidative insults

Understanding of pathways activated by neuroprotective NMDAR signalling is of great importance, with huge potential for developing therapeutic strategies to combat CNS injury or degeneration. Whilst the research presented in this thesis does not at every stage involve the increase of NMDAR activation, consideration of this signalling pathway is intrinsic to each chapter, which in turn describe:

- the requirement of synaptic activity in the neuroprotection conferred by PACAP treatment
- the hitherto uncharacterised activation of the antioxidant GSH pathway by NMDAR signalling
- the augmentation of the GSH antioxidant pathway through stimulation of astrocytes with CDDO-F3

As will be discussed in the epilogue of this thesis, each shed new light on the neuroprotective pathways of the CNS, which ultimately may provide new therapeutic strategies to counter injury and degeneration of the brain.

Chapter 2
Materials and Methods

Neuronal cultures

Cortical neurons from E21 Sprague Dawley rats were cultured using the method described (Bading and Greenberg, 1991; McKenzie et al., 2005a). Tissue culture grade plastic was incubated for at least two hours at 37 °C in poly-D-lysine (molecular weight 30,000 – 70,000) and Laminin (Sigma). Embryos were anaesthetised with an intraperitoneal injection of sodium pentobarbital (Merial Animal Health) and decapitated. Brains were removed, corticies dissected and placed in dissociation medium (81.8 mM Na_2SO_4, 30 mM K_2SO_4, 5.84 mM $MgCl_2$, 252 μM $CaCl_2$, 1 mM HEPES, 0. 1% Phenol Red, 20 mM Glucose, and including 1 mM Kyurenic acid to prevent excitotoxic death via NMDARs during dissection and enzymatic digestion). Once the required amount of tissue had been isolated, the corticical hemispheres were placed in a tube, excess liquid removed and incubated for twenty minutes at 37 °C in dissociation medium containing 10 units/ml of papain (Worthington Biochemical Corporation). This digestion step was repeated once more after removal of media. Subsequently, corticies were washed twice in dissociation media, then twice in growth medium containing NeuroBasal-A Medium, B-27 Supplement, Anti-Anti Supplement (anti-bacterial/anti-mycotic) (Invitrogen), 1mM glutamine and 1% Rat Serum (Harlan SeraLab). After washing, corticies were triturated by rapid suction/expulsion using a 2 ml disposable plastic pipette in 10 ml of warm growth medium. This cell suspension was then diluted using Opti-MEM (Invitrogen) supplemented with Glucose (20 mM), to obtain a concentration of one cortical hemisphere per 14 ml cell suspension. This solution was then plated on the pre-coated tissue culture plastic, with 0.5 ml of cell suspension used per well of a 24-well plate. Culture plates were incubated at 37 °C in a humified 5% CO_2 atmosphere for two and a half hours, after which the cell suspension was removed and replaced with 1 ml of growth medium. At DiV 4, 1 ml of growth media supplemented with 9.6 μM cytosine β-D-arabinofuranoside hydrochloride (AraC) (Sigma) was added to prevent proliferation of glial cells. Pure neuronal rat cultures were prepared as above except anti mitotic AraC was added at Div 0. Normal rat neuronal cultures treated with AraC at Div 4 contain ~5% GFAP positive cells (Papadia et al., 2008), whilst early AraC treatment leads to cultures containing <0.05% GFAP positive astrocytes

(Fig. M1). Astrocytes were cultured as described (Soriano et al., 2008b) by plating mixed neuronal/glial cultures at half density in 10% FBS containing DMEM (Invitrogen) without anti-mitotic. PKA RIIβ knockout mice (Watson et al., 2006), Nrf2 knockout mice (Chowdhry et al., 2010) and Keap1 knockout mice (Wakabayashi et al., 2003) and their respective wildtypes were cultured as above from E17 animals. Experiments were performed after a culture period of 9-10 days during which neurons developed a rich network of processes, expressed functional NMDA and AMPA/kainate glutamate receptors, and formed synaptic contacts (Hardingham et al., 2001b).

Stimulations and assessment of nuclear morphology

Prior to stimulations, neurons were transferred from growth medium to a trophically deprived medium (TMo) containing 10% MEM (Invitrogen), 90% Salt-Glucose-Glycine (SGG) medium (SGG (Papadia et al., 2005): 114 mM NaCl, 0.219 % NaHCO3, 5.292 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 10 mM HEPES, 1 mM glycine, 30 mM glucose, 0.5 mM sodium pyruvate, 0.1 % Phenol Red; osmolarity 325mosm/l) and allowed to equilibrate before stimulation. To quantify cell death, neurons were fixed and subjected to DAPI (Vectorlabs) staining and cell death quantified by counting (blind) the number of apoptotic nuclei as a percentage of the total. Approximately 1500 cells were counted per treatment. Morphologically, peroxide-treated and trophically deprived neurons show typical signs of apoptotic-like cell death (shrunken cell body and large round chromatin clumps). GFAP immunocytochemistry was performed by incubating fixed cells in 1:1000 Rabbit anti GFAP (Sigma) overnight, incubating 1 h each in 1:200 biotinylated anti-rabbit IgG and 1:500 Cy3-conjugated streptavidin (Jackson Immuno) before subsequent DAPI staining. Images were taken using a Leica AF6000 LX imaging system, with a DFC350 FX digital camera.

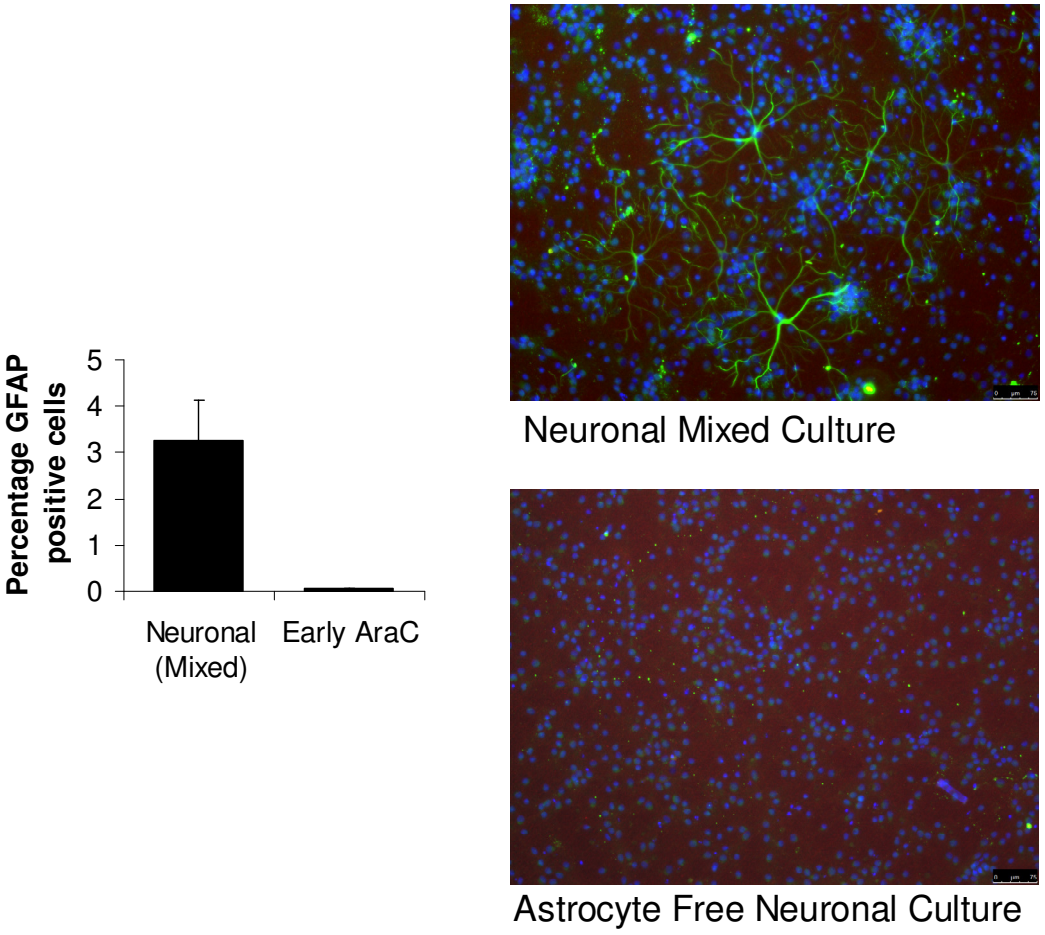


Figure M1. Early AraC treatment blocks proliferation of astrocytes in rat primary neuronal culture. (*Right)* Example pictures showing immunocytochemistry to GFAP (green) and NeuN (red) with nuclear DAPI stain (blue) of (*Upper)* regular Div 5 AraC treated rat cortical cultures and (*Lower*) Div 0 AraC treated cultures. **(*Left*)** Quantification of percentage of GFAP positive cells from either preparation (n = 6 cultures, with 8 10x magnification pictures taken, with approxiamtely 900 DAPI stained nuclei per picture).

For stimulations the following reagents were used: PACAP-27 was purchased from NeoMPS, MK801, KN-62, forskolin, BSO and BCNU from Tocris, bicuculline, D3T, MK571 and hydrogen peroxide from Sigma, PD-98059 from Ascent Scientific, H-89 from LC Laboratories, staurosporine, TTX, GSH and 4-aminopyridine from Calbiochem, γglutamylcysteine and cysteinylglycine from Bachem. CDDO-EA and CDDO-F3 were a kind gift from Professor Sporn.

Calcium Imaging

For preconditioning experiments neurons were treated as indicated for 2 h, then transferred to aCSF (150 mM NaCl, 3 mM KCl, 10 mM HEPES, 2 mM CaCl2, 1mM MgCl2, 1mM glucose) similarly conditioned. Ca2+ imaging was performed as described (Soriano et al., 2008c). Briefly, cells were loaded with 11μM Fluo-3 AM (Invitrogen) (from a stock solution of 2.2 mM Fluo-3 dissolved in anhydrous DMSO containing 20% (w/v) Pluronic detergent) for 30mins at 37°C. Fluo-3 fluorescence images (excitation 488 nm, emission ≥ 515 nm) were taken at one frame per second. To calibrate images, Fluo-3 was saturated by adding 50 μM ionomycin to the perfusion chamber and quenched with 10mM MnCl2 + 50 μM ionomycin to levels corresponding to 100 nM Ca^{2+} (Minta et al., 1989). Free Ca^{2+} concentrations were calculated according to the equation $[Ca^{2+}] = Kd(F – Fmin)/(Fmax – F)$, where F is the fluorescence and expressed as a multiple of the Kd (which is approximately 315 nM).

Western Blotting

After stimulation cells were lysed in 45 μl of sample buffer (1.5 mM Tris, 15% glycerol, 3% SDS, 7.5% β-Mercaptoethanol, 0.0375% bromophenol blue, pH 6.8), and boiled at 100 °C for 4 minutes. Gel electrophoresis and Western Blotting was performed using the Xcell Surelock system (Invitrogen) with 4-20% NuPage BisTris pre-cast gels (Invitrogen). 15 μl of sample was loaded per well and electrophoresis was performed in running buffer (50 mM MOPS, 50 mM Tris, 1 mM EDTA, 3.5 mM SDS, pH 7.7) at 160 V for ~ 1.5 h. Post migration, proteins were transferred

onto a PVDF membrane (Millipore) in transfer buffer (96 mM glycine, 12 mM Tris and 20% Methanol) at 45 V for ~ 2 h. Once transferred, membranes were blocked in TBS solution (20 mM Tris, 137 mM NaCl and 0.1% Tween-20) supplemented with 5 % dried milk. The membrane was then incubated overnight at 4 °C with following primary antibody diluted in TBS + milk solution: Anti Phospho-p44/42 MAPK Thr202/Tyr204 (p-ERK1/2; 1:2000, Cell Signaling), p44/42 MAPK (ERK1/2; 1:2000, Cell Signaling), phospho-CREB ser133 (1:500, Upstate) and CREB (1:500, Upstate). After this incubation, membranes were washed 3 times for 5 min in TBS, and then placed with the appropriate horseradish peroxidase-conjugated secondary antibody diluted in TBS + milk solution at room temperature for 1 h. Membranes were washed a further 3 times in TBS, then incubated for 1 min in LumiGlo reagent and peroxide (Cell Signaling Technology). Chemiluminescence was then detected by exposing Kodak X-Omat film to the membrane; then quantified using band densitometry using ImageJ software. Band density was normalised each time to an appropriate loading control, obtained by stripping the membrane of antibodies by incubating in ReBlot Plus Strong Stripping Solution (Millipore) for 15 min, followed by 3x 5 min TBS washes, and recapitulating the protocol from initial primary antibody incubation.

Transfection and luciferase assays

Neurons were transfected at DIV8 using Lipofectamine 2000 as described (Mckenzie et al., 2005b) using a total of 0.6 µg cDNA/well and 2.33 µl/well of Lipofectamine 2000 (1 µg/ml, from Invitrogen). Under these conditions transfection efficiency is approximately 2-5 %, with >99% of eGFP-expressing transfected cells NeuN-positive, and <1% GFAP positive (Papadia et al., 2008; Soriano et al., 2008b, a) confirming their neuronal identity.

For CRE-reporter assays, neurons were transfected with 0.5 µg of CRE-Firefly Luciferase + 0.1 µg of pTK-Renilla (Promega); or 0.2 µg CRE-Luc, 0.1 µg Renilla and 0.4 µg of: β-globin control vector, pICER1 (a gift from Dr. Paulo Sassone-Corsi, Centre National de la Recherche Scientifique-Institut National de la Santé et de la

Recherche Médicale, Strasbourg, France (Stehle et al., 1993)), CRTC1 and Dominant negative CRTC1 (TORC1 and TORC1-N44 were a gift from Dr Yang Zhou Institute of Neuroscience, Chinese Academy of Sciences, Shanghai, China (Zhou et al., 2006) (Zhou et al., 2006)). For CBP-reporter assays, neurons were transfected with 0.4 μg of GAL4-CBP (Chawla et al., 1998), 0.2 μg of a Firefly Luciferase reporter containing four GAL4 DNA binding sites (GAL4-Luc, Promega), and 0.1 μg pTK-Renilla; or 0.2 μg GAL4-CBP, 0.2 μg GBD-Luc, 0.1 μg pTK-Renilla and 0.2 μg of vectors expressing either β-globin control or E1A (described (Bannister and Kouzarides, 1995)). For GSR-promoter reporter assays, the following constructs were used: GSR Full-promoter (a 2kb sequence upstream of the mouse GSR gene) and ARE2 (a 132 bp ARE containing sequence located within the larger sequence), both constructs cloned into pGL3 Basic luciferase vector (Harvey et al., 2009). Neurons were transfected with 0.5 μg of GSR Full-promoter and 0.1 μg pTK-Renilla; alternatively o.2 μg of GSR Full-promoter or ARE2 reporter, 0.1 μg pTK Renilla and 0.3 μg of β-globin control or TAM67 (described (Brown et al., 1993)).

Stimulations were performed 24 h post transfection. For CRE-luciferase assays neurons were treated with 10 nM PACAP or 5 μM Forskolin for 4 h, or with 50 μM bicuculline and 250 μM 4-aminopyridine for 8 h, with inhibitors added 1 h before. For CBP activity assays neurons were treated with 10 nM PACAP for 8 h. For GSR-promoter assays neurons were treated with 50 μM bicuculline, 250 μM 4-aminopyridine and 50 μM MK801 for 4 h and 8 h. All assays were performed using the Dual Glo assay kit (Promega) and were performed on a FLUOstar OPTIMA (BMG Labtech, Aylesbury, UK). Firefly luciferase activity was normalized to the Renilla control in all cases and each experiment was performed at least 4 times.

Following the fate of transfected cells

The overall method to do this is as described (Soriano et al., 2008a; Soriano et al., 2010) with some modifications to the timing. Neurons were transfected with 0.5 μg of vectors expressing β-globin or ICER1 plus 0.1 μg of peGFP (Invitrogen). Neurons were placed in trophically-deprived medium and treated with 10 nM PACAP after 24

h where indicated. After a further 24 h images were taken of GFP expressing neurons using a Leica AF6000 LX imaging system, with a DFC350 FX digital camera, prior to treatment of neurons with TTX to block AP firing. Using mark-and-find software, the fate of the photographed neurons was followed at 24 h and 48 h after TTX treatment. 250-400 cells were analysed per treatment across 6 cultures within 3 independent experiments.

CRTC1-localisation

For CRTC1-localisation neurons were transfected with 0.6 μg GFP tagged CRTC1 (peGFP-C2/TORC1 a gift from Dr Dong-Yan Jin, Department of Biochemistry, University of Hong Kong, Hong Kong China (Siu et al., 2006)). 24 h post transfection neurons were treated with 20 ng/ml Leptomycin B (LC Laboratories) for 30 min in order to visualise CRTC1 import more clearly (Kovacs et al., 2007) and then 10 nM PACAP for a further 30 min in the presence of 1 μM TTX, 10 μM H-89 or 10 μM FK-506 (added 1 h before). Neurons were then fixed and stained for anti-GFP (1:750, Invitrogen) and visualised using biotinylated secondary antibody/cy3-conjugated streptavidin. Nuclei were counter-stained with DAPI. Subcellular distribution of CRTC1 was scored as being nuclear if levels were higher in the nucleus than in the surrounding perinuclear cytoplasm. 400-800 cells were analysed per treatment across 4-8 independent experiments.

Electrophysiological recording and analysis

All electrophysiological recordings were kindly performed by Dr Marc-Andre Martel. Coverslips containing cortical neurons were transferred to a recording chamber perfused (at a flow rate of approximately 5 ml/min) with an external recording solution composed of (in mM): 150 NaCl, 2.8 KCl, 10 HEPES, 2 CaCl2, 1 MgCl2 and 10 glucose, pH 7.3 (320-330 mOsm). Patch-pipettes were made from thick-walled borosilicate glass (Harvard Apparatus, Kent, UK) and filled with a K-gluconate-based internal solution containing (in mM): 155 K-gluconate, 2 MgCl2, 10 Na-HEPES, 10 Na-PiCreatine, 2 Mg2-ATP and 0.3 Na3-GTP, pH 7.3 (300 mOsm).

Electrode tips were fire-polished for a final resistance ranging between 5-10 MΩ . Currents were recorded at room temperature (21 ± 2°C) using an Axopatch-1C amplifier (Molecular Device, Union City, CA) and stored on digital audio tape. Data was subsequently digitized and analyzed using WinEDR v6.1 software (John Dempster, University of Strathclyde, UK). Neurons were voltage-clamped at –70 mV, and recordings were rejected if the holding current was greater than –100 pA or if the series resistance drifted by more than 20% of its initial value (<35 MΩ). Neurons were treated ± PACAP (10 nM) for 1-2 h prior to spontaneous EPSCs being recorded in voltage-clamp for 5 min. Recordings were studied to determine whether they showed evidence of burst-like activity, defined as long periods of activity (>1 s), peaking at >50 pA.

Glutathione content assays

Total glutathione was recorded using two methods. For basal level recordings neurons were treated as indicated for 24 h; for glutathione depletion assays 24 h stimulation was then preceded by addition of 100 µM H_2O_2 (50 nmoles in 500 µl) and incubated 24 h, with subsequent peroxide stimulations 8, 6, 4 and 2 h prior to cell lysis. 30 min before cessation of stimulations 50 µM Monochlorobimane (MCB) (Sigma) was added to cultures. MCB forms a fluorescent product with GSH after conjugation by glutathione S-transferase (Shrieve et al., 1988). After MCB incubation neurons were washed once in warm TMo, example pictures were taken, then cells were lysed in 100 µl lysis buffer (50 mM K2PO4, Triton 0.5%, pH 7) then centrifuged at 15,700 g (13,000 rpm) at 4 °C for 10 min. 80 µl of supernatant was then transferred to a black 96-well plate and fluorescence intensity was measured (excitation 485 nm, emission 520 nm) with a FLUOstar OPTIMA (BMG Labtech, Aylesbury, UK). Fluorescence was normalised to protein levels, determined using a bicinchonic acid (BCA) protein assay (Pierce, Thermo Scientific).

Alternatively a colorimetric method was followed using a kit (Calbiochem). After stimulation cells were lysed 50 µl lysis buffer (50 mM K_2PO_4, Triton 0.5%, pH 7) then centrifuged at 15,700 g (13,000 rpm) at 4 °C for 10 min. 35 µl of supernatant

was then transferred to a centrifuge tube, and mixed with 35 µl of 50 mg/ml Metaphosphoric Acid, incubated on ice for 5 min then centrifuged at 580 g (2,500 rpm) at 4 °C for 5 min; which precipitates protein from the lysate (Perlmann and Herrmann, 1938). 60 µl of deproteinated supernatant was then mixed with 120 µl assay buffer (K_2PO_4, diethylenetriamine penta-acetic (DTPA) and lubrol). 20 µl of reagent 1 (4-chloro-1-methyl-7-trifluoromethyl-quinolinium methylsulphate) was added and the mixture thoroughly vortexed, then 20 µl of reagent 2 (30% NaOH) was add and vortexed again. Samples were incubated at room temperature for 10 min, then transferred to a clear 96-well plate and absorbance was measured at 405 nm with a FLUOstar OPTIMA (BMG Labtech, Aylesbury, UK). A standard curve of GSH (five serial dilutions rangingfrom 16 µM to 1 µM and a lysis buffer blank) was run in parallel to calibrate GSH concentration; whilst protein concentration was determined from remaining lysate using a BCA assay, to allow quantification of mg GSH per g of protein.

Glutamyl-cysteine ligase assay

To determine GCL activity neurons were treated as indicated for 24 h, and enzyme activity was recorded as described (White et al., 2003). Briefly, post-stimulation samples were lysed over ice in 650 µl lysis buffer (20 mM Tris, 1 mM EDTA, 250 mM sucrose, 20 mM sodium borate , 2 mM L-serine (borate and serine added to inhibit γ-glutamyl transpeptidase activity (Tate and Meister, 1978) that may otherwise degrade reaction products), pH 7.4), then centrifuged at 15,700 g (13,000 rpm) at 4 °C for 10 min. Supernatants were then redistributed to 50 µl aliquots, one for each time point, and placed at 37 °C in a heat block. For buthionine sulfoximine samples, 15 µl of 100 mM BSO was added to 285 µl of control supernatant and thoroughly mixed by vortex before redistribution. 50 µl of GCL reaction buffer (400 mM Tris, 40 mM L-glutamic acid, 2 mM EDTA, 20 mM sodium borate, 2 mM L-serine, 40 mM $MgCl_2$, 40 mM ATP (added fresh to buffer during sample centrifugation), pH 7.4) was pipetted to each sample and allowed to incubate for 5 min. Reaction was started by adding 50 µl of 20 mM cysteine (dissolved in ATP-free reaction buffer) to samples and incubated for 20, 15, 10 and 5 minutes. The

GCL reaction was stopped by adding 50 µl of Metaphosphoric acid (2.5 g per 100 ml) to precipitate protein; subsequently, cysteine was added to zero time-points, samples were vortexed and placed on ice for 20 min. After incubation, samples were centrifuged at 580 g (2,500 rpm) at 4 °C for 5 min. Following centrifugation, 20 µl of reaction mixture (with at least 2 technical repeats per sample) was transferred to a black 96-well plate, and 180 µl of detection buffer (50 mM Tris (pH 10), 0.5 N NaOH and 10 mM 2,3-Napthalenedicarboxyaldehdye (NDA) (Sigma) (v/v/v – 1.4/0.2/0.2)) was added to each well. The NDA in this mixture rapidly forms a fluorescent cyclic reaction product with the cysteine thiol and glutamyl amino groups of GSH and GCγ ($\lambda_{excitation\ max}$ = 472 nm, $\lambda_{emission\ max}$ = 528 nm) (Orwar et al., 1995). The plate was left to incubate in the dark at room temperature for 30 min, and fluorescence intensity was measured (excitation 485 nm, emission 520 nm) with a FLUOstar OPTIMA (BMG Labtech, Aylesbury, UK). GCL activity was determined by calculating rate of fluorescence increase over time, normalised to protein content.

Glutathione Reductase Assay

To determine glutathione reductase activity NADPH oxidation was recorded as a loss of absorbance at 340 nm as described (Carlberg et al., 1985). Post stimulation cells were lysed in 50 µl of lysis buffer (20 mM HEPES pH 7.9, 100 mM KCl, 300 mM NaCl, 10 mM EDTA, 0.1% Nonidet P-40) and centrifuged at 15,700 g (16,400 rpm) at 4 °C for 10 min. Subsequently 20 µl of sample was distributed to a well of a clear 96-well plate, with 20 µl of 1 mM GSSG and 110 µl of assay buffer (100 mM Potassium Phosphate, pH 7.0), with a 0.1 units/ml solution of yeast glutathione reductase (Sigma) as a positive control and lysis buffer as a negative control. Using a multichannel pipette 50 µl of 1 mM NADPH was added to each well starting the reaction. NADPH absorbance was then read every 30 s for 15 min using FLUOstar OPTIMA (BMG Labtech, Aylesbury, UK). GR activity was determined by calculating rate of NADPH loss over time, normalised to protein content.

Glutathione Peroxidase Assay

To determine glutathione peroxidise activity a kit was used (Calbiochem) following the method described (Flohe and Gunzler, 1984). Post stimulation cells were lysed in 50 µl of lysis buffer (20 mM HEPES pH 7.9, 100 mM KCl, 300 mM NaCl, 10 mM EDTA, 0.1% Nonidet P-40) and centrifuged at 15,700 g (16,400 rpm) at 4 °C for 10 min. Subsequently 20 µl of sample was distributed to a well of a clear 96-well plate, with 70 µl of assay buffer (50 mM Tris-HCl, pH 7.6, 5mM EDTA), 50 µl of co-substrate mixture (NADPH, glutathione and glutathione reductase), with glutathione peroxidase as a positive control and lysis buffer as a negative control. Using a multichannel pipette 20 µl of cumene hydroperoxide was added to each well starting the reaction. NADPH absorbance was then read every 30 s for 15 min using FLUOstar OPTIMA (BMG Labtech, Aylesbury, UK). GPX activity was determined by calculating rate of NADPH loss over time, normalised to protein content.

RNA isolation, RT-PCR and Quantitative PCR

RNA was isolated using the Roche isolation reagents including a 15 min DNase I treatment to avoid genomic DNA contamination of samples, and eluted using 50 µl of RNase-free water. For qPCR cDNA was synthesised from 1-5 µg of RNA with the Tanscriptor One-Step RT-PCR Kit (Roche). Briefly, 7 µl of RNA was mixed on ice with 10 µl 2x cDNA Synthesis master mix, random primers: oligo primers 2:1 (total 3 µl), 2 µl deoxynucleotide mix (1 mM each: dATP, dTTP, dCTP and dGTP), 0.5 µl RNase Inhibitor (40 U/µl), 0.5 µl Reverse Transcriptase (20 U/µl) and 3 µl nuclease free water. Reaction mixtures were vortexed and spun down and run in parallel with at least one NoRT control. Samples were placed in a thermal cycler and incubated for 10 min at 25 °C, 30 min at 55 °C, 5 min at 85 °C, and were then cooled down to 4 °C.

This cDNA was then diluted to 6 ng per µl for use in real-time quantitative PCR. qPCR was performed in an Mx3000P qPCR System (Stratagene) using 2x FastStart Universal SYBR Green Master Mix (Roche) according to the manufacturer's

instructions. Briefly a total volume of 15 µl qPCR reaction mix was added per well, containing 1 µl template DNA, 7.5 µl SYBR Green master mix (containing ROX), 0.6 µl of forward and reverse primers at 200 nM final concentration and 5.3 µl nuclease free water. Technical replicates, NoRT controls and template-free controls were included in each case. The cycling program was 10 min at 95 °C; 40 cycles of 30 s at 95 °C, 40 s at 60 °C with detection of fluorescence and 30 s at 72 °C; followed by one cycle of 1 min at 95 °C, 30 s at 55 °C ramping up to 95 °C over 30 s with continuous fluorescence detection (for dissociation curve). Expression of the gene interest was calculated using the efficiency corrected ΔΔCt method (Livak and Schmittgen, 2001), normalising to either GAPDH or 18s as a housekeeping gene. The sequence and efficiencies of primers used are shown below, with primers specific for both rat and mouse unless otherwise specified.

mRNA Target	Efficiency (%)	Forward Primer	Reverse Primer
Gapdh Rat	106	AGAAGGCTGGGGCTCACC	AGTTGGTGGTGCAGGATGC
GCLC Rat	88.3	CCAACCACCCAACCCTCTG	TGCTCTGGCAGTGTGAATCC
Gapdh Mouse	104	GGGTGTGAACCACGAGAAAT	CCTTCCACAATGCCAAAGTT
GCLC Mouse	107	CCAACCATCCGACCCTCTG	TGTTCTGGCAGTGTGAATCC
18s	94.7	GTGGAGCGATTTGTCTGGTT	CAAGCTTATGACCCGCACTT
GCLM	90.7	GCACAGCGAGGAGCTTC	GAGCATGCCATGTCAACTG
GSS	99.8	TCAGATTACATGTTCCAGTGC	GCACGCTGGTCAAATATG
GSR	88.5	GGCATGTCATCAAGGAGAAG	TGGGATCTGGTTCTCATGAG
GPX1	81.4	GGAGAATGGCAAGAATGAAG	AAATGATGTACTTGGGGTCG
GPX2	88	GCCTCAAGTATGTCCGCCCTG	GGAGAATGGGTCGTCATAAGG
GPX4	88	GGAGCCAGGAAGTAATCAAG	ACCATAGCGCTTCACCAC

Cysteine Uptake assays

Cysteine uptake assays were kindly performed by Dr Kunal Gupta. Cortical cultures stimulated for 24 h in TMo with 50 µM bicuculline and 250 µM 4-aminopyridine, 50 µM MK801 or 250 nM CDDO-F3. Samples were washed with PBS, and incubated with 10 µM ^{35}S-cysteine (containing 2 µM dithiothreitol) at for 10 min at 37 °C. Uptake was stopped by placing samples on ice. Cells were then washed twice in ice-cold PBS and radioactivity measured using a scintillation counter.

Statistical analysis

All results are presented as mean ± standard error of the mean. Statistical testing involved a 2-tailed paired student T-test. For studies employing multiple testing, we used a one-way ANOVA followed by Fisher's LSD post-hoc test.

Chapter 3
PACAP induces long lasting neuroprotection through the induction of activity dependent signalling via CRTC1

Introduction

Due to its neuroprotective properties both *in vitro* and *in vivo* and its ability to cross the blood brain barrier, the neurotrophic peptide PACAP has received considerable attention as a potential therapeutic agent (Somogyvari-Vigh and Reglodi, 2004; Shioda et al., 2006; Brenneman, 2007; Ohtaki et al., 2008; Vaudry et al., 2009). It is generally assumed that PACAP-mediated PKA signalling in neurons triggers neuroprotective gene expression and signal pathways by direct modulation of upstream effectors of these processes. However, there is an alternative explanation: that PACAP-induced PKA signalling exerts at least some of its neuroprotective effects indirectly though the enhancement of electrical activity. G-protein coupled receptors that activate cAMP/PKA signals in neurons, such as type I mGluRs and D1-type dopamine receptors, can potentiate synaptic strength, neuronal excitability and ion channel properties (Nguyen and Woo, 2003). PACAP administration has been recently reported to enhance AMPAR currents as well as synaptic NMDAR currents (MacDonald et al., 2007; Costa et al., 2009) and to suppress the apamin-insensitive slow after-hyperpolarization (IsAHP) current (Hu et al., 2010), which can control neuronal excitability.

Physiological patterns of synaptic activity are known to be strongly neuroprotective (Bell and Hardingham, 2011b), activating multiple pathways including CREB-mediated gene expression, antioxidant gene expression and the suppression of apoptotic genes (Hetman and Kharebava, 2006; Al-Mubarak et al., 2009; Hardingham and Bading, 2010; Soriano and Hardingham, 2011; Zhang et al., 2011b). An episode of activity can confer neuroprotection long after that episode has ceased, via a mechanism involving the activation of nuclear Ca^{2+}- and CREB-dependent gene expression (Papadia et al., 2005; Zhang et al., 2009). Microarray analysis of genes influenced by synaptic activity revealed an NMDAR dependent upregulation of PACAP, which was confirmed by qPCR (Papadia et al., 2008; Martel et al., 2009b), consistent with its transcriptional regulation by CREB (Fukuchi et al., 2004; Miyashita et al., 2005). Thus, we wished to study the effect of PACAP on

levels of electrical activity in cortical neurons, and the role this plays in neuroprotection.

We found that PACAP-induced PKA signalling in cortical neurons is by itself insufficient to activate neuroprotective pathways; instead the anti-apoptotic effect of PACAP stimulation is mediated indirectly, through the induction of synchronised action potential (AP) firing. Treatment of rat cortical neurons with PACAP induced a rapid and sustained PKA-dependent increase in AP firing and associated Ca2+ transients. Exposure to PACAP protected neurons from apoptosis inducing staurosporine treatment, and activated protective ERK1/2 signalling; however these effects were both blocked in the presence of tetrodotoxin (TTX). Transient exposure to PACAP induced a long-lasting neuroprotection which was reliant on AP firing and efficient CREB mediated gene expression, a phenomenon we have previously observed in neurons that have undergone a period of increased synaptic activity (Papadia et al., 2005). Whilst activity independent PKA signalling was sufficient to phosphorylate CREB on its activating serine-133 site, further secondary-activating steps by activity dependent CaM kinase and calcineurin are required for full CRE-dependent gene transcription. PACAP induced transactivation of CREB binding partner CBP was demonstrated to be dependent on AP firing and subsequent activation of CaM kinase. PACAP also induced nuclear import (and thus activation) of a further CREB coactivator CRTC1, through firing activity dependent stimulation of calcineurin signalling. Overexpression of CRTC1 was sufficient to rescue PACAP induced CRE-dependent gene expression in the presence of TTX. Thus the enhancement of AP firing may play a significant role in the neuroprotective actions of PACAP and other adenylate cyclase coupled signalling molecules. These results were recently published (Baxter et al., 2011).

Results

PACAP triggers sustained increases in AP firing in cortical neurons.

PACAP is known to promote PKA-dependent neuroprotection in a variety of systems *in vitro* and *in vivo*. However, PKA activation is also capable of altering neuronal network activity through the control of intrinsic excitability and synaptic strength (Nguyen and Woo, 2003). To investigate the effect of PACAP on levels of electrical activity we performed Ca^{2+} imaging experiments on cortical neurons pre-treated with PACAP. This pre-treatment resulted in enhanced AP firing as evidenced by strong oscillatory intracellular Ca^{2+} transients that were blocked by the Na^{+} channel antagonist TTX (Fig. 1.1A). In contrast, control neurons exhibited far smaller TTX-sensitive Ca^{2+} transients (Fig. 1.1B). Pre-treatment of neurons with the PKA inhibitor H-89 prevented any PACAP-induced changes in Ca^{2+} oscillations (Fig. 1.1C). We also performed whole-cell voltage-clamp recordings of neurons pre-treated with PACAP which corroborated the Ca^{2+} imaging data: nine out of nine PACAP-treated neurons exhibited incoming excitatory post-synaptic currents consistent with burst-like activity (>1 s in duration, > 50 pA at peak), compared to 0 out of 5 control neurons (Fig. 1.1E). Further Ca^{2+} imaging experiments revealed that acute administration of PACAP also had a similar effect, indicating that the potentiating effect of PACAP on AP firing is fast-acting as well as long-lasting (Fig. 1.1F). Thus, PACAP-induced PKA signalling in cortical neurons induces long-lasting increases in AP firing and associated Ca^{2+} transients.

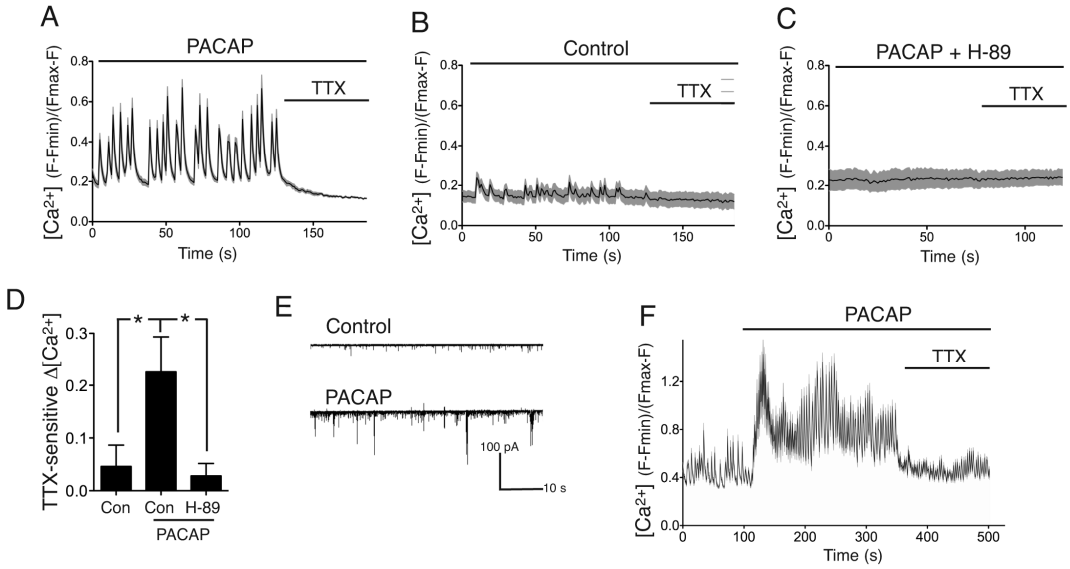


Figure 1.1. PACAP enhances AP firing in cortical neurons. (A - D) Pre-treatment with PACAP (10 nM here and throughout the study) causes an increase in AP firing-dependent Ca2+ transients. Neurons were treated where indicated with 10 nM PACAP ± H-89 (10 µM). After 2 h the neurons were subjected to Fluo-3 Ca2+ imaging studies (see methods for details) to monitor the size of Ca2+ transients in the different stimulation conditions. TTX (1 µM) was added where indicated to determine the extent to which the observed Ca2+ transients were due to action potential firing. Example traces are shown: black line indicates the mean Ca2+ concentration within a field of cells, and the grey shaded region indicates ± s.e.m. of the Ca2+ concentration within that field. Free Ca2+ concentrations were calculated from fluorescence signal (F) according to the equation [Ca2+] = Kd(F – Fmin)/(Fmax – F), and expressed as a multiple of the Kd of Fluo-3 (which is approximately 315 nM). **(D)** shows quantification of data shown in 1a-c, i.e. quantification of the difference in mean amplitude of [Ca2+] before and after 1 µM TTX treatment. In order to quantitate the effect of PACAP on firing activity-induced Ca2+ influx, the mean [Ca2+] 30 seconds before and 30 seconds after TTX treatment was calculated in either control neurons or neurons treated with PACAP ± H-89. For each cell, the degree of TTX-sensitive Ca2+ changes was calculated as the difference between mean [Ca2+] before and after TTX treatment. For each condition, 60 cells were analysed within 6 independent experiments (*P<0.05). **(E)** Example trace of a whole-cell voltage-clamp recording of a control and PACAP-treated cortical neurons. PACAP causes an increase in burst-like activity, consistent with the Ca2+ imaging data. **(F)** Ca2+ imaging of acute PACAP treatment, a typical example trace is shown representative of 6 independent experiments.

Enhanced AP firing is essential for PACAP-induced neuroprotection.

It is known that elevated electrical activity can promote neuroprotection in cortical neurons (Al-Mubarak et al., 2009; Bell and Hardingham, 2011b), raising the possibility that PACAP-induced AP firing might contribute to its neuroprotective effect. We studied the capacity of PACAP to protect neurons against two different apoptotic insults, and studied the effect of blocking AP firing by TTX treatment. We first used staurosporine which induces caspase-dependent apoptosis of cortical neurons (Papadia et al., 2005). Pre-treatment of cortical neurons with PACAP before exposure to staurosporine for 24 h reduced levels of apoptosis (Fig. 1.2A-B). TTX treatment alone enhanced basal levels of neuronal apoptosis, however staurosporine treatment caused additional neuronal loss. Significantly, PACAP treatment failed to protect neurons against apoptosis in the presence of TTX.

We next employed a model of prolonged trophic deprivation (72 h) that also induces progressive caspase-dependent apoptosis (Papadia et al., 2005). Once again, PACAP treatment protected neurons against apoptosis in control, although overall levels of apoptosis were not high (Fig. 1.2C, treatment (1)). An episode of AP firing can promote neuroprotection that lasts well beyond the point at which that activity ends (Papadia et al., 2005). We hypothesised that PACAP-induced AP firing would similarly be able to exert long-lasting neuroprotection. Neurons subjected to trophic deprivation were treated with or without PACAP for 24 h, after which all neurons were placed in PACAP-free, TTX-containing medium. Levels of apoptosis were then assessed after a further 48 h. PACAP treatment was found to confer significant neuroprotection (Fig. 1.2C, treatment (3)) and this was dependent on PACAP-induced AP firing, since no protection was observed if PACAP was administered in the presence of TTX (Fig. 1.2C, treatment (2)). Thus, PACAP-induced AP firing confers long-lasting neuroprotection. We conclude from these experiments that PACAP-induced enhancement of AP firing is important for its neuroprotective effects in these models of cortical neuronal apoptosis. This suggests that activation of PKA signalling is insufficient to *directly* activate certain neuroprotective

pathways: it activates them *indirectly* by inducing AP firing which in turn triggers Ca^{2+}-dependent signalling pathways that induce pro-survival events.

AP firing is required for PACAP-induced ERK activation

To study the molecular events required for PACAP-mediated neuroprotection we investigated activation of the ERK1/2 pathway, which is known to mediate some of the acute neuroprotective effects of PACAP (Falluel-Morel et al., 2004; Li et al., 2005; Stumm et al., 2007)**.** Consistent with previous studies, PACAP treatment results in strong activation of ERK1/2 which is blocked by PKA inhibition with H-89 treatment (Fig. 1.2D). Moreover, inhibition of ERK1/2 activity by treatment with the MEK1 inhibitor PD98059 exacerbated apoptosis induced by trophic deprivation, and rendered PACAP unable to offer any protection, even when trophic deprivation was limited to 48 h (Fig. 1.2E). We then looked at whether activation of ERK1/2 by PACAP required AP firing and found that it did: TTX prevented PACAP-induced activation of ERK1/2 (Fig. 1.2F), indicating that PACAP-induced PKA signalling does not activate ERK1/2 directly, but activates it indirectly by promoting firing.

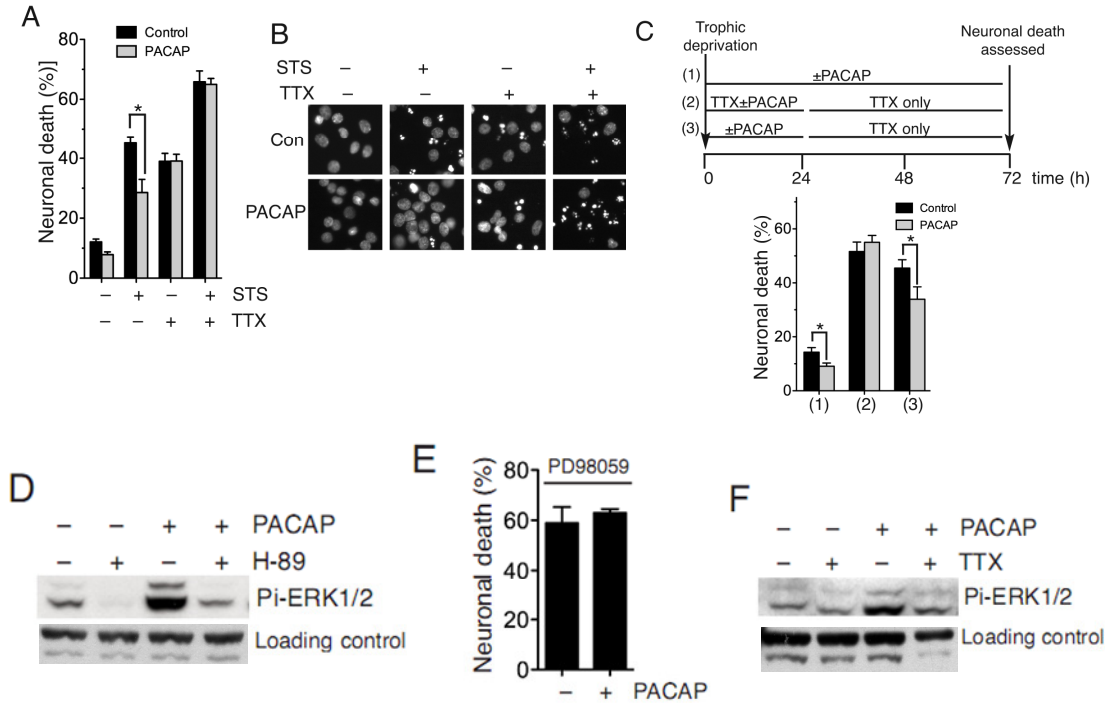


Figure 1.2. PACAP promotes resistance to apoptotic stimuli which is dependent on AP firing. (A,B) PACAP protects against staurosporine-induced cell death, but not in the presence of TTX. Neurons were treated with PACAP in the presence or absence of TTX 24 h and 1 h before treatment with 100 nM staurosporine (STS). After a further 24 h, the cells were then fixed and DAPI stained and death was measured by counting pyknotic and non-pyknotic nuclei (*P<0.05. n = 4 (here and throughout, n indicates data from separate cultures, with cell death measured from 4 pictures taken from each of 2 replicate wells; with approximately 1500 cells counted per treatment each time)); **(B)** shows example pictures. **(C)** PACAP-induced AP firing protects against trophic deprivation and promotes long lasting neuroprotection. At t=0, the neurons were placed in trophically-deprived medium and given one of the three treatment regimes outlined in the upper schematic (1-3). At t=72 h, cells were fixed, DAPI stained and levels of neuronal death analysed (*P<0.05, n=3). **D)** PACAP induced ERK 1/2 activation is blocked by PKA inhibition. Neurons were treated with PACAP ± 10 μM H-89. Cell lysates were obtained and analysed for phospho-ERK1/2, normalised to CREB protein expression (n = 4, example blot shown). **E)** ERK1/2 pathway inhibition blocks PACAP neuroprotection. Neurons were treated with PACAP for 15 min in the presence or absence of 50 μM PD98059 (added 1 h prior to PACAP), with cell death analysed 48 h later (n = 4). **F)** PACAP-induced ERK 1/2 activation is blocked by TTX. Neurons were treated for 15mins with PACAP ± 1μM TTX (n = 4, example blot shown).

Induction of CREB-mediated gene expression contributes to PACAP-mediated neuroprotection.

We next investigated the mechanism by which PACAP-induced AP firing leads to long-lasting neuroprotection. The CREB family controls the expression of a number of pro-survival genes containing CRE promoter elements and is a target for activation by both cAMP/PKA signals as well as activity-dependent Ca^{2+} signalling (Lonze and Ginty, 2002). CREB itself is the predominant member in forebrain neurons (Papadia et al., 2005) and so is likely to be responsible for the majority of CRE-mediated gene expression. PACAP treatment resulted in the strong activation of a CRE-reporter raising the possibility that CREB activation contributes to PACAP-mediated neuroprotection (Fig. 1.3A). We studied the effect of blocking CRE-dependent gene expression by transfecting neurons with a vector encoding ICER1, which is an inhibitory isoform of the CREB family (De Cesare and Sassone-Corsi, 2000). We confirmed the efficacy of ICER1: expression of ICER1 blocked PACAP-induction of a CRE-reporter gene (Fig. 1.3A).

To assess the importance of CREB in PACAP-induced long-lasting protection, neurons were transfected with vectors expressing either β-globin (control) or ICER1 plus a peGFP transfection marker. At 24 h post-transfection, neurons were placed in trophically-deprived medium and treated ± 10 nM PACAP. After a further 24 h images were taken of GFP-expressing neurons, prior to the transfer of the neurons to PACAP-free, TTX-containing medium to block AP firing. The fate of the transfected neurons was followed at 24 h and 48 h after TTX treatment (see Fig. 1.3B schematic). We found that the transfection procedure caused slightly higher rates of neuronal death than in untransfected cells. However, in control-transfected neurons, PACAP treatment promoted significant protection both 24 h and 48 h after the removal of PACAP (Fig. 1.3B). Importantly, PACAP treatment was not significantly neuroprotective in ICER1-expressing neurons (Fig. 1.3B), indicating a role for CRE/CREB-dependent gene expression in PACAP-mediated long-lasting neuroprotection.

AP firing underlies PACAP-induced CREB activation

The fact that PACAP-induced neuroprotection is not observed when neurons are co-treated with TTX suggested that activation of CRE-dependent gene expression could be dependent on AP firing. Indeed, we found this to be the case: TTX treatment alone had little effect on basal activity of a CRE- reporter, but inhibited PACAP-mediated activation by around 80% (Fig. 1.3C). PKA inhibition by H-89 treatment completely blocked the induction of the CRE reporter by PACAP, *including* the small TTX-insensitive component. Taken together, these data show that direct signalling by PKA is able to support weak activation of CRE-dependent gene expression, but that AP firing is needed for strong CRE-induction and resultant neuroprotection.

To further confirm the role of PKA in both activity-dependent and -independent activation of CREB by PACAP, we studied activation of a CRE reporter in neurons cultured from a mouse deficient in the RIIß subunit of PKA. In the RIIß$^{-/-}$ mouse, levels of cAMP-inducible PKA activity within the cortex are lower than wild-type, while basal PKA activity is similar (Brandon et al., 1998). We found that both TTX-sensitive and -insensitive components of PACAP-induced CRE-mediated gene expression were lower in RIIß-null neurons (Fig. 1.3D). The level of reduction in PACAP-induced CRE-activation in the RIIß-null neurons was comparable to that seen in the context of the adenylate cyclase activator forskolin (Fig. 1.3D), confirming that PKA is central to both AP-dependent and -independent components of CREB activation by PACAP.

Given that activity-dependent Ca^{2+} influx can activate Ca^{2+}-dependent adenylate cyclases, it was theoretically possible that PKA could play a role in CREB activation *downstream* of AP firing. However, we have previously shown that strong firing activity does not cause global levels of cAMP to rise sufficiently high to support PKA signalling to CREB in the nucleus (Pokorska et al., 2003). Nevertheless, to investigate this directly we studied the activation of CRE-mediated gene expression by AP firing induced via a PKA-independent mechanism: network disinhibition by

the $GABA_A$ receptor blocker bicuculline plus the K^+ channel blocker 4-aminopyridine (to enhance burst frequency (Hardingham et al., 2001a)). Induction of CRE-mediated gene expression by B/4AP-induced AP firing was *not* lower in RIIß-null neurons (Fig. 1.3D). This indicates that cAMP/PKA signalling is not a major mediator of CRE-dependent gene expression *downstream* of AP firing, in agreement with previous studies (Pokorska et al., 2003). Collectively these observations support a model whereby PACAP-induced PKA signalling weakly activates CREB directly, but triggers strong CREB activation by promoting AP firing which in turn activates CREB via PKA-independent pathways.

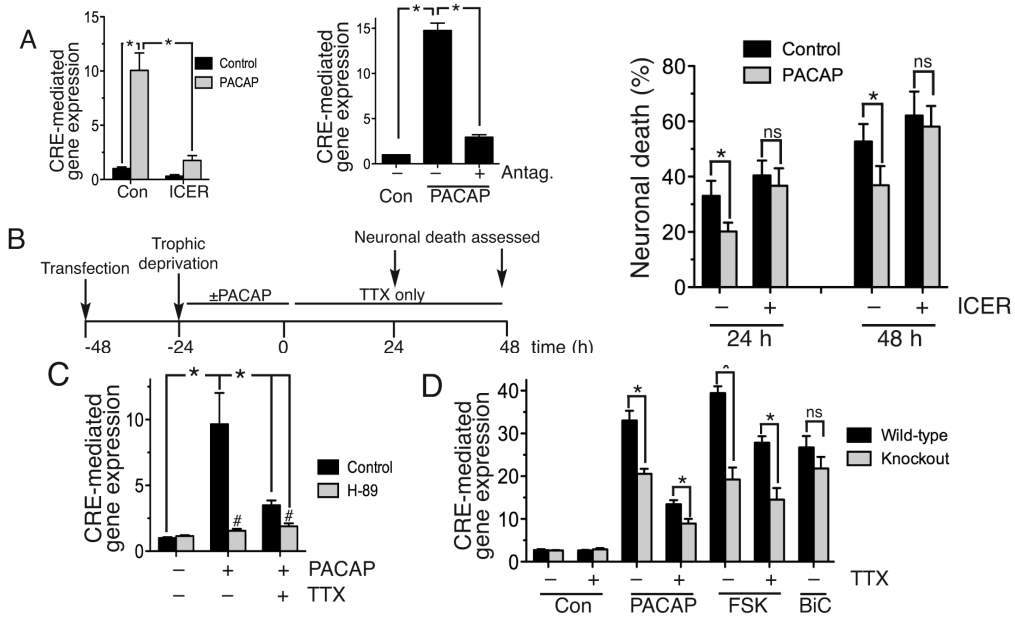


Figure 1.3. PACAP induces CRE-dependent gene expression, which is neuroprotective, and relies on AP firing. (A) PACAP induces CRE-mediated gene expression. Neurons were transfected with a CRE-Firefly luciferase vector, pTK renilla transfection control and vectors encoding either ICER1 or control (β-globin). At 24 h post transfection, neurons were treated with PACAP and luciferase expression was measured after a further 4 h CRE-Firefly luciferase activity was normalised to Renilla control (*P<0.05, n = 3) *Right* Effect of the PACAP antagonist (Antag. PACAP6-38, 1 μM) on PACAP induction of CRE-luciferase (*P<0.05, n = 3). **(B)** PACAP mediated long-lasting neuroprotection depends on activation of CRE-mediated gene expression. Left panel illustrates the experimental protocol. Briefly, neurons expressing GFP plus either ICER1 or β-globin control were treated ± PACAP 24 h post-transfection and then all cells were placed in TTX-containing medium after a further 24 h, at which point images of GFP-expressing neurons were taken (t=0 in the upper schematic). The fate of these cells was then monitored at 24 and 48 h after this medium change. 250-400 cells were analysed per treatment across 6 cultures within 3 independent experiments. (*P<0.05). **(C)** PACAP induced CRE-dependent gene expression is dependent on AP firing. Neurons were treated with PACAP where indicated for 4 h; all other drugs were added 1 h beforehand (*P<0.05, n=7). **(D)** PACAP and forskolin-induced activation of CRE-mediated gene expression is disrupted in RIIβ-deficient neurons: both AP firing-dependent and independent components. Forskolin was used at 5 μM. For comparison is an illustration of the RIIβ-*independence* of CRE activation triggered by promoting AP firing by network disinhibition through treatment with the GABAA receptor blocker bicuculline (50 μM) plus 250 μM 4-aminopyridine, which is a PKA-independent way of inducing AP firing (*P<0.05, n=6).

PACAP-induced CBP, but not CREB phosphorylation, requires AP firing.

We next investigated which CRE-activating molecular events triggered by PACAP treatment are reliant on activity-dependent Ca^{2+} signals, and whether any can be triggered in an activity-independent manner by direct PKA signalling. CREB phosphorylation on serine-133 is essential for CREB activation since it triggers the recruitment of the coactivator CREB binding protein (CBP (Chrivia et al., 1993)). CREB phosphorylation was induced by PACAP treatment (Fig. 1.4A). Interestingly, TTX treatment did not interfere with PACAP-induced CREB phosphorylation (Fig. 1.4B), whilst the PKA inhibitor H-89 blocked CREB phosphorylation (Fig. 1.4A). Serine-133 of CREB is a good substrate for PKA (Gonzalez and Montminy, 1989), and these data indicate that PACAP-induced PKA activity is sufficient to result in the direct phosphorylation of CREB, and that AP firing is *not* needed for this particular activation step.

However, CREB serine-133 phosphorylation is necessary but not sufficient for full activation of CREB. Key secondary activation steps centre around CREB coactivators CBP and CRTC. CBP is recruited to serine 133 phospho-CREB and is itself activated by nuclear CaM kinase activity (Chawla et al., 1998; Hardingham et al., 1999; Impey et al., 2002). To study activation of CBP in isolation we tested the ability of CBP, tethered to the promoter by means of fusion to the GAL4 DNA-binding domain (DBD), to confer inducibility upon PACAP stimulation to a luciferase reporter containing four GAL4 sites. GAL4-CBP mediates strong activation of the reporter gene, compared to the GAL4 DBD alone (Fig. 1.4C). Furthermore, PACAP strongly activated CBP-mediated gene expression (Fig. 1.4C). To confirm the importance of CBP in activation of CREB by PACAP, we studied the effect of expressing the viral oncoprotein E1A, which inhibits CBP-mediated gene expression by directly interacting with it (Bannister and Kouzarides, 1995). E1A expression strongly inhibited GAL4-CBP-mediated gene expression (Fig. 1.4D) and also inhibited the activation of CRE-mediated gene expression (Fig. 1.4E). Thus, PACAP activates CBP, an important coactivator of CREB-dependent gene expression. Since CBP is subject to activity-dependent transactivation via CaM

kinase activity, which contributes to Ca^{2+}-activation of CRE-mediated gene expression (Hardingham et al., 1999; Impey et al., 2002), we wanted to know whether PACAP also triggered CBP and CRE activation by this route. Indeed, PACAP activation of CBP was TTX-sensitive, and was also inhibited by treatment with the CaM kinase inhibitor KN-62 (Fig. 1.4F). Moreover, KN-62 inhibited PACAP-induced activation of the CRE-reporter, an effect that was occluded by blockade of AP firing with TTX (Fig. 1.4G). This is consistent with the activity-dependent, but not independent, components of CRE-induction being mediated partly by CaM kinase activity, potentially via the established route of CBP activation. However, the inhibitory effect of KN-62 on PACAP-induced CRE activation was not as strong as that of TTX (Fig. 1.4G), indicating that other activity-dependent pathways are also important.

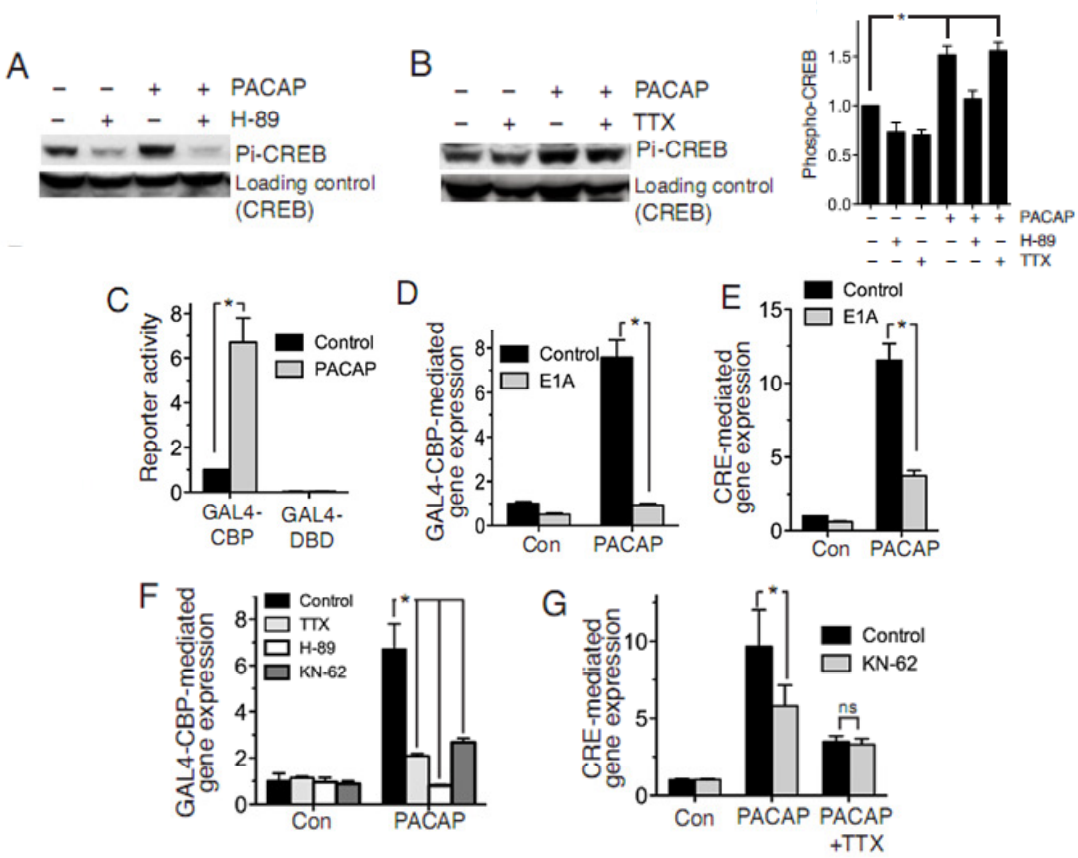


Figure 1.4. CBP activation, but not CREB phosphorylation, require PACAP-induced AP firing. A, B) PACAP induces phosphorylation of CREB at serine-133 in a TTX-insensitive, PKA-dependent manner. Neurons were pre-treated with TTX or H-89 and then treated for 15 min with PACAP (example blots are shown) *Right* Quantitation of phospho-CREB levels normalised to total CREB (*P<0.05, n = 4). **C)** PACAP stimulation induces CBP-mediated gene expression. Neurons were transfected with GAL4-luciferase reporter, pTK Renilla and either expression vector encoding a GAL4-CBP fusion protein, or just the GAL4 DNA binding domain (GAL4 DBD). At 24 h post transfection, neurons were stimulated with PACAP for 4 h, and luciferase expression was measured and normalised to Renilla control, *P<0.05 (n = 4). **D)** The viral oncoprotein E1A inhibits PACAP mediated CBP activation. Neurons were transfected with GAL4-Luciferase, pGAL4-CBP, pTK-Renilla and vectors encoding either E1A or β-globin control. Neurons were stimulated with PACAP for 4 h; P<0.05* (n = 4). **E)** E1A inhibits PACAP mediated increase in CRE-dependent gene expression. The effect of E1A expression on the PACAP-induced activation of CRE-luciferase was measured. *P<0.05 (n=4). **F)** PACAP-induced activation of GAL4-CBP-mediated gene expression measured in the presence of the indicated inhibitors, added 1 h prior to PACAP. *P<0.05 (n=5). KN-62 was used at 10 μM. **G)** CaM kinase activity contributes specifically to the AP firing-dependent component of PACAP-mediated CRE induction. Neurons were treated as indicated and PACAP-mediated activation of the CRE reporter measured as before after 4 h. *P<0.05 (n=4).

PACAP-induced AP firing mediates calcineurin-dependent CRTC1 nuclear import

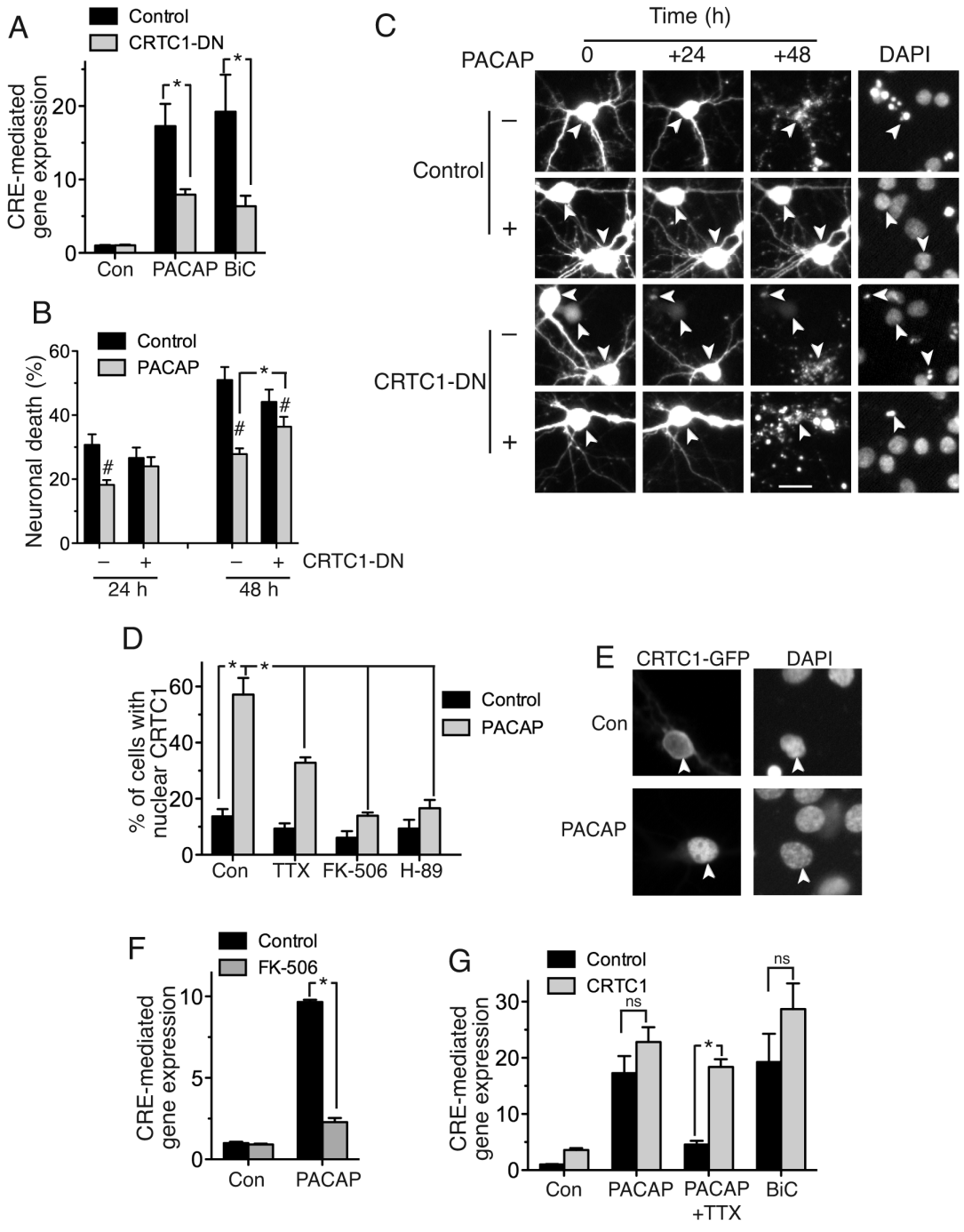
Whilst CREB serine-133 phosphorylation is necessary for full activation of CREB, it is not sufficient. A key secondary activation step involves the coactivator CRTC (CREB-regulated transcription coactivator), which is subject to Ca^{2+}-dependent nuclear import, where it binds to CREB and enhances its affinity for both CBP and the basal transcriptional machinery (Conkright et al., 2003; Screaton et al., 2004; Zhou et al., 2006; Kovacs et al., 2007; Li et al., 2009).

We confirmed the importance of CRTC for CRE activation: expression of a dominant negative mutant of CRTC1 (CRTC1-DN, (Zhou et al., 2006)) strongly inhibited PACAP-induction of the CRE-mediated gene expression, as well as that induced by B/4AP treatment (Fig. 1.5A). We also investigated the importance of CRTC signalling in PACAP-mediated long-lasting neuroprotection, using an identical protocol to that used in Fig. 1.3B, except that the ICER-encoding vector was replaced with that of CRTC1-DN. At the 48 h timepoint (after removal of PACAP from the medium) a very small, but statistically significant, amount of PACAP-dependent neuroprotection was still observed at 48 h in CRTC1-DN-expressing neurons. However, levels of neuronal death in CRTC1-DN-expressing neurons previously exposed to PACAP were significantly higher than control-transfected cells previously exposed to PACAP (Fig. 1.5B,C). Thus, CRTC1-DN interferes with neuroprotection evoked by transient exposure to PACAP, consistent with the role of CREB in this process, and the importance of CRTCs in CREB-mediated gene expression.

Nuclear translocation of CRTC is an important step in the full activation of CREB-dependent gene expression (Screaton et al., 2004). We found that PACAP treatment caused the nuclear translocation of CRTC that was inhibited by TTX (Fig. 1.5D,E). Activity-dependent Ca^{2+} influx is known to induce CRTC translocation through activation of the Ca^{2+}-dependent phosphatase calcineurin. Calcineurin subsequently dephosphorylates CRTC, triggering its nuclear import and coactivation of CREB (Li

et al., 2009). This mechanism is employed in the case of PACAP signalling: inhibition of calcineurin activity by treatment with the inhibitor FK-506 inhibited PACAP-mediated CRTC translocation (Fig. 1.5D) and PACAP-mediated induction of CRE-mediated gene expression (Fig. 1.5F).

Taken together, these observations suggest that a key reason why TTX inhibits PACAP-activation of CRE-mediated gene expression is that blockade of CRTC nuclear import renders nuclear levels of CRTC too low to efficiently coactivate CREB. We postulated that if CRTC was indeed limiting, then if we overexpressed CRTC, then this might rescue the inhibitory effect of TTX on PACAP activation of the CRE reporter. Although overexpressed CRTC would be mainly cytoplasmic, we reasoned that since a proportion of it is nuclear then this could rescue the deficiency in nuclear levels. We found this to be the case: overexpression of CRTC reversed the inhibitory effect of TTX on PACAP-induction of CREB-dependent gene expression (Fig. 1.5G). CRTC overexpression, however, did not further enhance PACAP activation of CREB-mediated gene expression in the absence of TTX (Fig. 1.5G), indicating that PACAP-induced firing causes sufficient CRTC nuclear import such that levels of nuclear CRTC are not limiting for efficient coactivation of CREB. Thus, while PACAP activation of direct PKA signalling is sufficient to induce CREB phosphorylation, this is insufficient to activate CREB on its own. Enhancement of AP firing is critical in order to induce calcineurin-dependent CRTC nuclear translocation, and important step in CREB activation and consequent neuroprotection.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PACAP +TTX
BiC

Figure 1.5 PACAP induces nuclear translocation of CRTC1, necessary for the AP firing-dependent component of CREB activation. **(A)** CRTC1 dominant negative inhibits PACAP mediated activation of CREB. Neurons were transfected with CRE-luciferase, pTK-Renilla and vectors encoding either a CRTC1 dominant negative mutant or control (b-globin). Neurons were stimulated PACAP or bicuculline plus 4-AP (BiC) (*p < 0.05, n = 4). **B, C)** PACAP mediated long-lasting neuro-protection depends on CRTC1. The experimental protocol is the same as that illustrated schematically in Fig. 3(b). Briefly, neurons expressing GFP plus either CRTC1-DN (dominant negative) or b-globin control were treated ± PACAP 24 h post-transfection and then all cells were placed in TTX-containing medium after a further 24 h, at which point images of GFP-expressing neurons were taken. The fate of these cells was then monitored at 24 and 48 h after this medium change (*p < 0.05, paired T-test, n =3; #p < 0.05, paired T-test comparing control to PACAP within each condition/timepoint). **(C)** shows example pictures. Scale bar = 20 μm. **(D ,E)** PACAP induces CRTC1 nuclear translocation via activity-dependent calcineurin signalling. Neurons were transfected with a vector encoding GFP-tagged CRTC1. At 24 h post-transfection, neurons were treated with 20 ng/mL leptomycin B for 30 min to block nuclear export [to enable import to be observed more clearly (Kovacs et al. 2007)], plus the indicated inhibitors (1 lM TTX, 10 lM H-89 or 10 lM FK-506) and then PACAP added for 30 min prior to fixing of the cells and analysing localisation of GFP-CRTC1 in 400–800 cells per treatment (*p < 0.05, n = 4–8). **(E)** shows example pictures. **(F)** PACAP-induced activation of CRE-mediated gene expression requires the Ca2+ -dependent phosphatase calcineurin. Where used, FK-506 was added 1 h prior to PACAP stimulation (*p < 0.05, n = 4). **(G)** CRTC1 over-expression rescues the inhibition of PACAP-mediated CRE activation by TTX. Neurons were transfected with CRE-luciferase, pTK-Renilla and either vectors encoding CRTC1 or b-globin control. 24 h post-transfection the neurons were stimulated with PACAP ± TTX or bicuculline + 4-AP (BiC) as indicated. Over-expression of CRTC1 does not further enhance CRE activation by BiC or PACAP, suggesting that levels are not limiting, however, it strongly enhances levels induced by PACAP in the presence of TTX (*p < 0.05, n = 4).

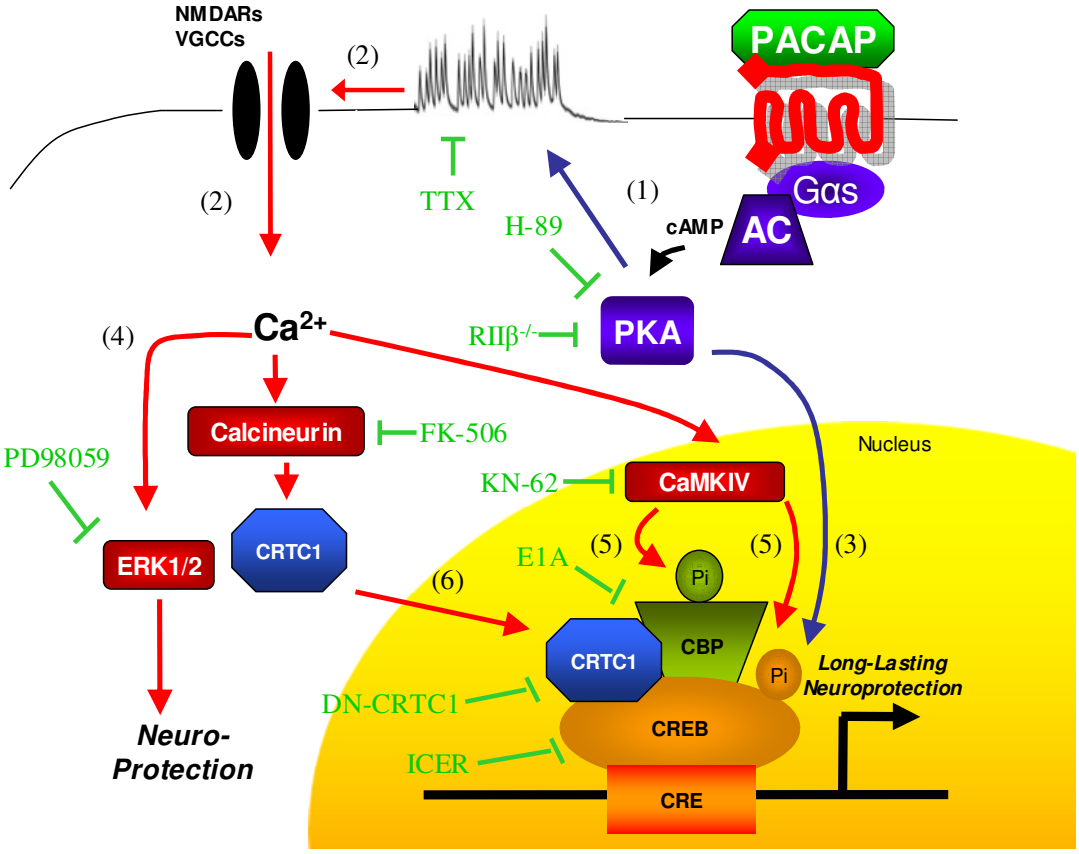

Figure 1.6. Schematic illustration of the role of activity-dependent Ca2+ signalling in PACAP-mediated neuroprotection. Activation of PACAP receptors leads to activation of PKA via the classical G-protein-adenylate cyclase (AC)-cAMP (pathway 1). PKA activation causes an increase in synaptic strength and/or neuronal excitability leading to a strong increase in levels of action potential firing which in turn triggers intracellular Ca2+ influx, likely through synaptic receptors (e.g. NMDA receptors) or voltage-gated Ca2+ channels (VGCCs) (pathway 2). Activation of long-lasting neuroprotection by PACAP requires induction of gene expression mediated by the transcription factor CREB. CREB phosphorylation on serine-133 can be triggered directly by PKA in an AP firing-independent manner (pathway 3). However, this is insufficient to fully activate CREB-mediated gene expression. Ca2+/activity-dependent pathway activates neuroprotective ERK1/2 kinases (pathway 4); and CamKIV which phosphorylates both CREB and CBP (pathway 5). A key synaptic activity dependent component of PACAP stimulated gene expression involves CRTC1 nuclear translocation through activation of the Ca2+-dependent phosphatase calcineurin: pathway (6). Blue arrows and molecules indicate AP firing activity-independent events, while red arrows and molecules highlight the events dependent on AP firing. The pharmacological and genetic inhibitors of the various pathways used in this study are shown in green.

Discussion

This study shows that certain PACAP-mediated anti-apoptotic signals in cortical neurons are not mediated by direct cAMP/PKA-dependent activation. Instead, the primary role of cAMP/PKA signalling is to enhance neuronal network activity. The resulting AP-dependent Ca^{2+} transients are the direct activators of neuroprotection, and induce a long-lasting phase of protection dependent on activation of CREB-mediated gene expression. These events are illustrated schematically in Fig. 1.6.

Modulation of neuronal electrical activity by PACAP and other AC-coupled ligands.

The ability of PACAP to induce AP firing in networks of cortical neurons is consistent with the known influence of intracellular cAMP on neuronal excitability. Neurotransmitters, neuropeptides and pharmacological compounds that activate AC are well-known to modulate neuronal excitability, ion channel conductance, and synaptic transmission and plasticity, predominantly through PKA activation (Nguyen and Woo, 2003). At the synapse, pharmacological activators of AC, and agonists of AC-coupled receptors such as the D1/D5 dopamine receptor, or the ß-adrenergic receptor all mimic LTP and/or enhance EPSPs (Nguyen and Woo, 2003). Mice deficient in AC1 and AC8 show deficits in LTP and spatial memory (Nguyen and Woo, 2003; Ferguson and Storm, 2004). At the molecular level, PKA-mediated GluR1 phosphorylation at serine-845 increases AMPA receptor open probability and stabilizes synaptic location of AMPA receptors trafficked to the synapse during LTP (Banke et al., 2000; Esteban et al., 2003; Lee et al., 2003). PACAP at low doses is known to enhance AMPAR currents via PKA activation (Costa et al., 2009) as well as synaptic NMDAR currents (MacDonald et al., 2007). Moreover, mice deficient in PACAP have defective LTP at the mossy fibre synapse, implicating endogenous PACAP signalling in synaptic potentiation (Otto et al., 2001). In addition to modifying the properties of synaptic glutamate receptors, AC-coupled PKA signalling also can modulate neuronal excitability by controlling the IsAHP. IsAHP is mediated by a Ca^{2+} activated potassium current which is activated in response to

bursts of AP firing. This is a key negative regulator of neuronal excitability, inducing a prolonged state of hyperpolarization, and this is in turn negatively regulated by AC-coupled PKA activity induced either pharmacologically (e.g. forskolin) or by treatment with AC-coupled ligands (e.g. dopamine) (Pedarzani and Storm, 1995; Lancaster et al., 2006; Hu et al., 2010). PACAP treatment itself leads to inhibition of IsAHP in cortical pyramidal neurons (Hu et al., 2010), which could contribute to the enhanced AP firing that we observe (Fig. 1.1).

PACAP-induced AP firing promotes CREB-dependent neuroprotection

The CREB family of transcription factors is known to be an important mediator of activity-dependent gene expression (Lonze and Ginty, 2002). Activation of CREB-mediated gene expression requires serine-133 phosphorylation which is necessary to recruit CBP to CREB (Chrivia et al., 1993). Several Ca^{2+}-activated kinase cascades can mediate this event, including the Ras-ERK1/2 pathway and also nuclear CaM kinases (Soriano and Hardingham, 2007). However, PACAP induced CREB phosphorylation does not require these activity-dependent pathways, since even when AP firing is blocked CREB phosphorylation is still observed (Fig. 1.4). This is consistent with the fact that PKA is also a CREB kinase and indicates that PACAP-induced PKA activity is strong enough to mediate this event directly.

However, direct PKA activity induced by PACAP is not sufficient to induce subsequent activation steps, including nuclear translocation of CRTCs. CRTCs enhance the interaction of CREB with the TAF(II)130 component of TFIID following its recruitment to the promoter (Conkright et al., 2003). CRTC also plays an important role in assisting the recruitment of CREB's coactivator CBP to phosphorylated serine-133 CREB (Ravnskjaer et al., 2007). Calcineurin promotes nuclear translocation of CRTC2 through calcineurin-mediated dephosphorylation of serine-171 (Screaton et al., 2004). Translocation can be enhanced/synergized by PKA signalling with causes the inhibition of the serine 171 -kinase– salt-inducible kinase-2 SIK2 (Screaton et al., 2004). CRTC1 is the major isoform in the brain and is a key regulator of CREB-dependent gene expression (Kovacs et al., 2007; Li et al.,

2009). Ca^{2+} signals promote the nuclear translocation of CRTC1, dependent on calcineurin signalling which directly dephosphorylates CRTC1 (Bittinger et al., 2004). Analogously with CRTC2, cAMP signals can also trigger the translocation of CRTC1 (Bittinger et al., 2004), most likely through the inhibition of SIK-mediated phosphorylation. In neurons, calcineurin activation is sufficient to trigger CRTC1 translocation (Li et al., 2009). The requirement for AP firing and calcineurin signalling for PACAP treatment to induce CRTC1 translocation strongly indicates that PACAP-induced PKA activity is not strong enough on its own to promote sufficient CRTC1 translocation directly, although may be playing a supporting role.

A caveat of some the data presented here is the reliance of a single drug to suggest the involvement of a particular enzyme, which runs the risk of reporting false positives due to non-specific actions of the drug. The PKA inhibitor H-89 for instance exhibits a moderately inhibitory effect on protein kinase G with an IC50 concentration 10-fold higher than that required for PKA (Chijiwa et al., 1990), with lesser effects on other kinases; thus cannot be referred to as a specific inhibitor of PKA activity. Whilst we are confident that the results are due to the loss of PKA activity, especially given in context of the entire body of work, use of a single drug can limit our conclusions. An example of such is our extrapolation of the inhibition of PACAP induced CRE-mediated gene expression by the CaM kinase inhibitor KN-62 (Fig. 1.4G). Whilst this likely due to the inhibitory effect of KN-62 on CaMKIV, as constitutively active CaMKIV but not CaMKII has been shown to increase CRE-mediated gene expression (Chawla et al., 1998); we cannot be certain of the identity of the specific CaM kinase blocked, since both are inhibited by the drug with very similar efficiencies (Enslen et al., 1994). This highlights the benefits of using multiple drugs, or multiple strategies such as siRNA knockdown or dominant-negative protein expression, to confirm the role of an enzyme.

PACAP prevents neuronal loss and dysfunction in vivo: potential role of enhanced AP firing.

PACAP has been reported to protect neurons against a variety of insults including ceramide, glutamate and hydrogen peroxide-induced death (Vaudry et al., 2009), insults that synaptic activity also protects against (Lee et al., 2005; Papadia et al., 2005; Papadia et al., 2008). Importantly, activation of CREB-mediated gene expression is implicated in activity-dependent protection against both apoptotic and excitotoxic insults (Lee et al., 2005; Papadia et al., 2005). Based on this study, it may be that indirect activity-dependent signalling to CREB contributes to the neuroprotective effects of PACAP *in vitro* and also begs the question as to whether any of its *in vivo* effects are similarly due to enhancing neuronal activity.

In vivo administration of PACAP reduces neuronal loss in the substantia nigra in acute models of Parkinson's disease: 6-OHDA and MPTP treatment (Reglodi et al., 2004; Reglodi et al., 2006). However, most neuroprotective studies on PACAP have centred on excitotoxic trauma: principally stroke and traumatic brain injury (TBI). PACAP crosses the blood brain barrier and can be administered intravenously to decrease damage in several models of ischemia and is effective even when administered several hours after the ischemic episode (Uchida et al., 1996; Reglodi et al., 2000; Chen et al., 2006; Ohtaki et al., 2008). Enhanced neuronal AP firing is known to protect neurons against excitotoxic cell death including ischemic conditions (Lee et al., 2005; Tauskela et al., 2008) and so the notion that PACAP can reduce neuronal damage in part by promoting AP firing is plausible. In addition to reducing stroke-induced damage, post-insult PACAP treatment also reduces the extent of axonal damage following TBI (Farkas et al., 2004; Tamas et al., 2006b). TBI is characterised by brief acute hyperactivity of ionotropic glutamate receptors, including NMDA receptors, which mediate acute excitotoxic damage, followed by sustained loss of function (Biegon et al., 2004; Yaka et al., 2007). As such the NMDA receptor has been proposed to rapidly switch between ‘destructive’ and ‘recovery’ roles (Biegon et al., 2004; Yaka et al., 2007). In the immature brain, treatment with NMDAR antagonists reduces primary excitotoxic death but

exacerbates secondary apoptosis, resulting in increased overall death (Pohl et al., 1999). By promoting AP firing, PACAP may boost the recovery phase post-injury by mechanisms related to those described in this study, as well as others more specific to the activity-dependent protection of axons.

Of course, enhanced neuronal activity is unlikely to mediate all the effects of PACAP in the CNS: direct activity-independent effects are likely to be exerted in neurons as well. Moreover, there are well-documented neuroprotective effects of PACAP acting indirectly via non-neuronal cells. For example, PACAP stimulates the astrocytic release of neuroprotective IL-6 (Ohtaki et al., 2008) and may also suppress microglial activation, thus reducing the release of potentially harmful cytokines that can form part of the post-ischemic response (Vaudry et al., 2009). Nevertheless, the impact of PACAP on neuronal activity should be taken into account when assessing the mechanism and extent of any therapeutic effect.

Chapter 4
Influence of synaptic activity on the glutathione antioxidant system

Introduction

Due to the longevity of neurons, their constitutive and inducible production of ROS, and their low expression of antioxidant enzymes (Mavelli et al., 1982; Halliwell, 1992; Yermolaieva et al., 2000); the dynamic regulation of antioxidant defences in neurons is vital. Increased synaptic activity may raise the demands for antioxidants, as this increases metabolic requirements and can induce enzymatic production of H_2O_2 (Boveris and Chance, 1973; Diaz-Hernandez et al., 2005; Brennan et al., 2009). The GSH antioxidant pathway displays greater expression in neurons than alternative H_2O_2 detoxifying systems (Mavelli et al., 1982), and both pharmaceutical or genetic blockade of GSH synthesis causes oxidative stress and neuronal death (Jain et al., 1991; Mizui et al., 1992; Diaz-Hernandez et al., 2005). GSH synthesis can be regulated post-transcriptionally by its feedback inhibition (Richman and Meister, 1975), and can be increased by supply of precursors from astrocytes (Dringen et al., 1999; Diaz-Hernandez et al., 2005; Vargas and Johnson, 2009). However GSH synthesis may also be controlled by its subunit mRNA expression, with upregulation of its catalytic subunit inducing increased GSH levels (Dickinson and Forman, 2002; Franklin et al., 2009). We considered the possibility that synaptic activity, particularly through stimulation of NMDARs, could drive changes in the GSH pathway through changes in gene expression.

We have recently observed that synaptic NMDAR activation promoted a neuroprotective response against oxidative insult, decreasing consequent intracellular ROS formation. This protection was partly mediated by coordinated changes in gene expression that enhanced the action of antioxidant enzymes Trx and Prx (Papadia et al., 2008). Due to the prevalence of the GSH pathway and its obvious importance in neurons, we wished to investigate if increased synaptic activity could cause similar enhancement of this antioxidant pathway.

We found that the protection conferred by increased synaptic activity against oxidative insult was lost in the presence of a GCL inhibitor; indeed GCL inhibition sensitised synaptically active neurons to sub-toxic H_2O_2 treatment compared to

controls. Whilst not increasing GSH levels in unstressed neurons, enhancement of synaptic activity led to a maintenance of GSH levels after oxidative insult, an effect dependent on GCL activity and NMDAR stimulation. In the presence of GCL and GR inhibitors, electrically active neurons displayed an increased metabolism of GSH, suggesting an increased GPX activity. Increases in GCL, GR and GPX enzymatic activity were observed in electrically active neurons; these were matched by changes in the mRNA expression of GCLC, GSR, GPX2 and GPX4. NMDAR blockade *in vivo* caused a decrease in GSH pathway enzyme mRNA expression and GCL activity after 12 h, with a subsequent loss of GSH after 24 h. These data suggest that NMDAR activation regulates the mRNA expression and thus the activity of the GSH antioxidant pathway, which may be intrinsic to the neuroprotective effect of NMDAR signalling against oxidative insult.

Results

Synaptic activity does not increase neuronal GSH levels, but requires its synthesis to mediate neuroprotection against oxidative insult

Having previously observed an induction of antioxidant defences in electrically active neurons, conferring a strong resistance against oxidative insult (Papadia et al., 2008), we wished to investigate the possible role of the GSH antioxidant pathway in this resistance. To do this, neurons were treated for 24 h in trophically deprived media with $GABA_A$ receptor antagonist bicuculline and K^+ channel blocker 4-aminopyridine (B/4AP), a treatment that in turn disinhibits the network by releasing excitatory neurons from tonic inhibition by GABAergic interneurons and inhibits neuronal hyperpolarisation after action potentials, leading to an increase in action potential firing and synaptic NMDAR Ca^{2+} currents (Hardingham et al., 2001b). B/4AP treatment caused a resistance against H_2O_2 treatment (Fig 2.1A), in accordance with previously observed results (Papadia et al., 2008). To investigate the role of the GSH pathway in this protection we blocked GSH synthesis with the potent GCL inhibitor buthionine sulfoximine (BSO), which binds to the cysteine accepting area of the GCL active site (Griffith and Meister, 1979). BSO treatment sensitised neurons to oxidative insult, causing pronounced neuronal death in response to the otherwise sub-toxic 50 μM dose of H_2O_2, and abolished the neuroprotective effect of B/4AP treatment (Fig 2.1A). Furthermore B/4AP treatment in the presence of BSO sensitised neurons to the lower dose of 25 μM H_2O_2 compared to similarly treated control neurons (Fig 2.1A), surprising given the observed resistance of synaptically active neurons to oxidative insult. We hypothesised that these contrasting results could be caused by an influence of synaptic activity on the GSH pathway. The GCL dependent protection by B/4AP treatment could be caused by a synaptic activity dependent increase in GSH synthesis or recycling. On the other hand, the sensitisation of electrically active neurons to sub-toxic oxidative insult in the presence of BSO could be explained by an increased GSH metabolism; in the absence of *de novo* GSH synthesis this would deplete GSH levels, which could in turn cause a sensitisation to oxidative insult.

To investigate GSH levels in electrically active neurons we employed a fluorescent probe MCB, which passes into cells and is conjugated to GSH in a reaction catalysed by glutathione S-transferase (Shrieve et al., 1988). No change in GSH levels was observed after 24 h treatment with B/4AP, nor after NMDAR blockade with MK801; however 24 h BSO treatment significantly decreased GSH levels (Fig 2.1B). This was recapitulated using an alternative colorimetric assay of GSH showing concordant results (Fig 2.1C). The reduction of neuronal GSH levels by GCL inhibition agreed with previous results (Ratan et al., 1994; Andersen et al., 1996; Bolanos et al., 1996; Diaz-Hernandez et al., 2005) that highlight the requirement of *de novo* synthesis for maintenance of GSH levels; furthermore this reduction in GSH explained the hypersensitivity of BSO treated neurons to oxidative insult. Whilst B/4AP treatment did not change GSH levels, we hypothesised that this did not rule out an influence of synaptic activity on GSH synthesis. GCL activity is inhibited in a non-allosteric fashion by GSH (Richman and Meister, 1975), which could obscure any increase in GSH synthesis capability in unstressed neurons. Accordingly, we decided to investigate the effect of increased synaptic activity on GSH depletion.

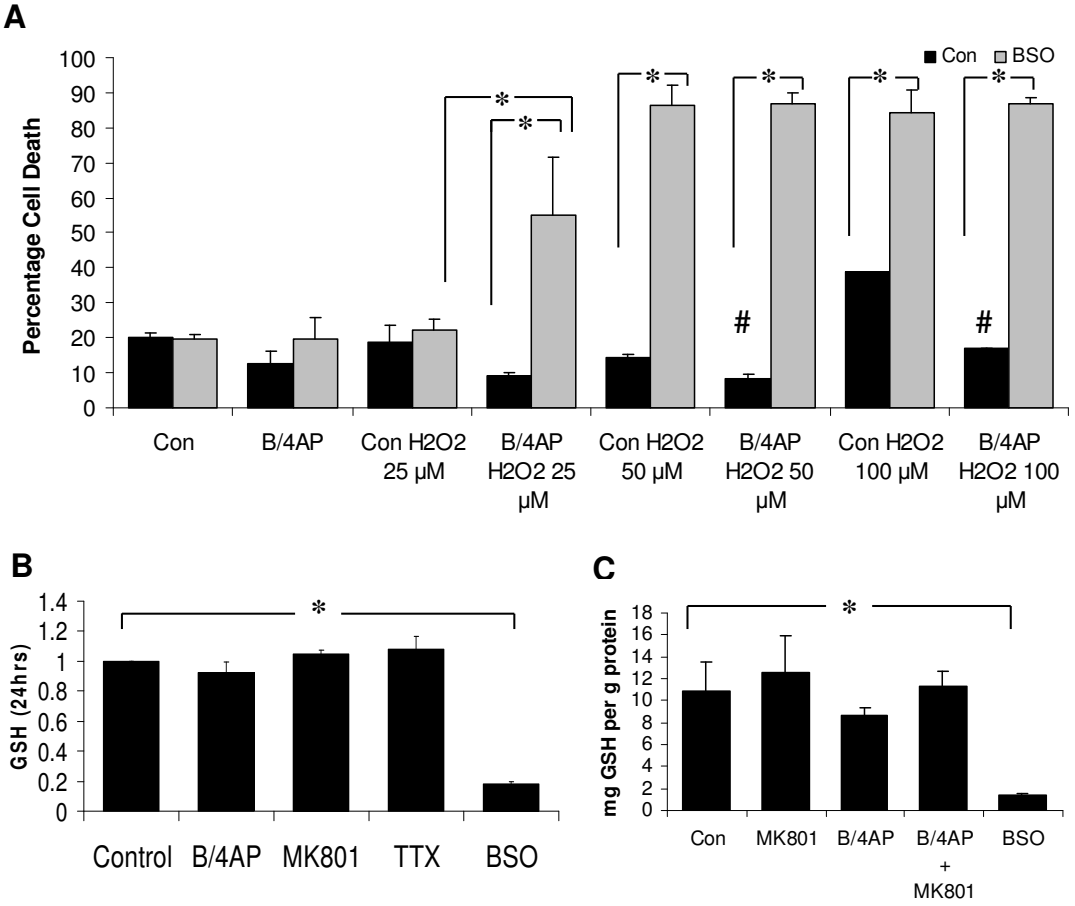

Figure 2.1. Inhibition of Glutathione synthesis blocks synaptic activity mediated neuroprotection. A) Analysis of rat cortical neuronal death. Neurons were placed in trophically deprived media and treated with 50 µM bicuculline and 250 µM 4-aminopyridine (B/4AP here and throughout) for 24 h in the presence with or without BSO (100 µM throughout), followed by overnight stimulation with concentration of H_2O_2 indicated, after which cells were fixed and DAPI stained. *$p<0.05$ compared to B/4AP + 25 µM H_2O_2, n=3. **B)** GSH levels measured using MCB fluorescent dye. Neurons were treated as indicated for 24 h, with 50 µM MCB, added 30 min before stimulation termination (in all MCB figures). Cells were then lysed, fluorescence recorded and normalised to protein content. Levels normalised to respective control, *$p<0.05$, n=4. **C)** GSH levels measured using colorimetric assay. Neurons were treated as indicated for 24 h, then lysates were probed for GSH, n=4.

Synaptic activity maintains GSH levels and inhibits its depletion in response to oxidative insult

Since the concentration of GSH in neurons is influenced by feedback inhibition, we investigated the effect of synaptic activity on GSH depletion. To do this, neurons were treated for varying lengths of time with H_2O_2 over at 24 h period, and their GSH levels were subsequently recorded using MCB (Fig 2.2A). Under these conditions, the rate of GSH depletion ($\frac{\Delta[GSH]}{\Delta t}$)is increased by the rate of GSH metabolism (m), and decreased by the rate of GSH recycling (r) and the rate of GSH synthesis (s). These can be expressed using the following equation:

$$\frac{\Delta[GSH]}{\Delta t} = m - (r + s)$$

Through selective inhibition of GSH pathway enzymes, we could then determine the possible effect of increased synaptic activity on the rate of each of these processes. Thus:

$$\frac{\Delta[GSH]}{\Delta t}_{BSO} = m - r$$

$$\frac{\Delta[GSH]}{\Delta t}_{BSO + BCNU} = m$$

By comparing depletion rates of neurons treated in parallel we could determine rate of synthesis:

$$\frac{\Delta[GSH]}{\Delta t} - \frac{\Delta[GSH]}{\Delta t}_{BSO} = s$$

Whilst B/4AP treatment of neurons had no effect on GSH levels in unstressed conditions, the resistance of electrically active neurons to oxidative insult made us

hypothesise that these neurons might maintain higher GSH levels in stressed conditions. Using the above method we found that $\frac{\Delta[GSH]}{\Delta t}$ was reduced in B/4AP treated neurons: whilst control neurons displayed GSH depletion over time in response to H_2O_2 stimulation, electrically active neurons maintained their GSH levels throughout (Fig 2.2B,C). When neurons were treated with BSO concurrent with H_2O_2, $\frac{\Delta[GSH]}{\Delta t}$ increased in both control and B/4AP treated neurons. The difference in depletion caused by BSO was greater in B/4AP treated neurons than controls:

$$\left(\frac{\Delta[GSH]}{\Delta t} - \frac{\Delta[GSH]}{\Delta t}_{BSO}\right)^{Control} < \left(\frac{\Delta[GSH]}{\Delta t} - \frac{\Delta[GSH]}{\Delta t}_{BSO}\right)^{B/4AP}$$

Thus;

$$(s)^{Control} < (s)^{B/4AP}$$

suggesting that increased synaptic activity causes an increased GSH synthesis in response to oxidative insult (Fig 2.2D). Importantly, $\frac{\Delta[GSH]}{\Delta t}_{BSO}$ was still significantly larger in controls than in B/4AP treated neurons (Fig 2.2C). Thus, these data suggested that the resistance to oxidative stress in electrically active neurons is mediated in part by an increased enzymatic activity of GCL.

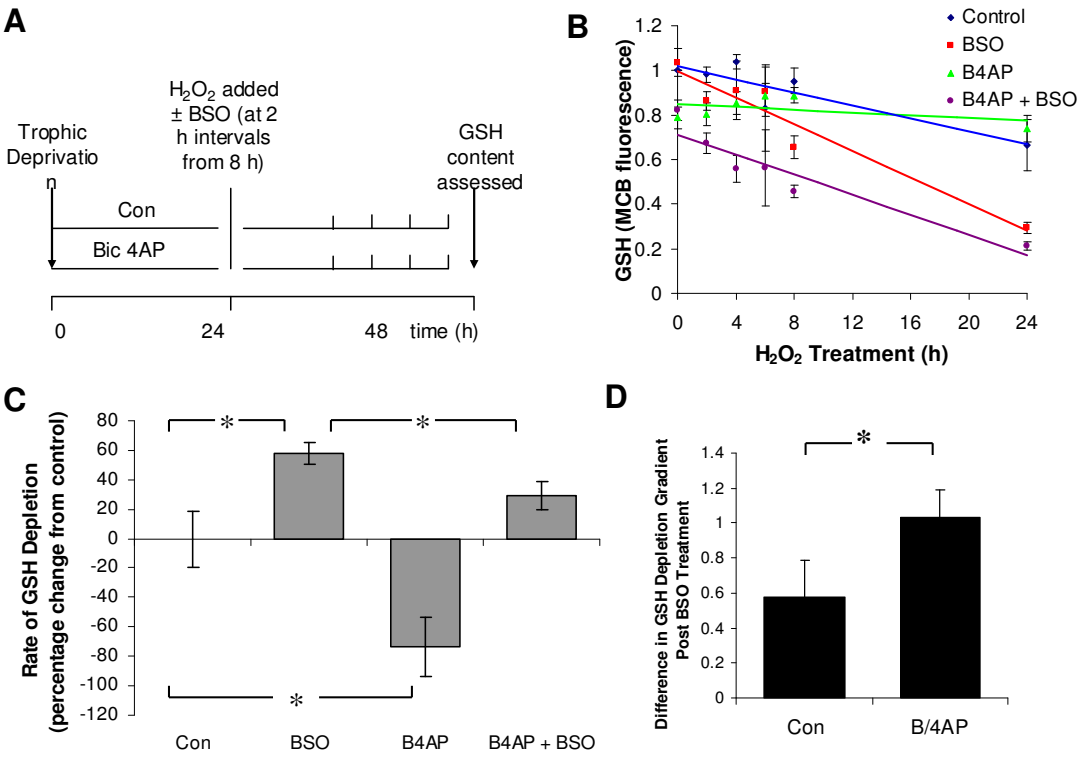

Figure 2.2. Synaptic activity inhibits GSH depletion. **A)** Schematic of experiment. After 24h in trophically deprived media, control and B/4AP treated neurons were stimulated with 100 µM H_2O_2 ± 100 µM BSO between 24 and 0 h, followed by MCB treatment and subsequent recording of fluorescence from cell lysates, which was normalised to protein content and respective control. **B)** Graph showing average GSH levels at each time point and derived slope indicating rate of GSH depletion. **C)** Analysis of GSH depletion gradient of neurons in the presence of H_2O_2. Each experiment is normalised to their respective control. **D)** Difference in GSH depletion of neurons between control and BSO treatments (n = 7, *p < 0.05).

Synaptic activity increases GSH metabolism

Taking into account the neuroprotective effect of B/4AP treatment, the increased cell death caused by low dose H_2O_2 treatment observed in neurons treated concurrently with B/4AP and BSO was intriguing. We considered that this apparent contradiction could be explained by an increased metabolism of GSH, which would reduce GSH levels in the absence of *de novo* synthesis, thus sensitising neurons to sub-toxic oxidative insult. To test this we measured GSH depletion in the presence of BSO and GR inhibitor carmustine (bis-chloroethylnitrosourea (BCNU)) (Fig 2.3A). The presence of these two inhibitors was sufficient to deplete neuronal GSH over an 8 h period, the rate of which was increased by concurrent stimulation with H_2O_2 (Fig 2.3B). We observed that:

$$\left(\frac{\Delta[GSH]}{\Delta t}_{\text{BSO + BCNU}}\right)^{\text{Control}} < \left(\frac{\Delta[GSH]}{\Delta t}_{\text{BSO + BCNU}}\right)^{\text{B/4AP}}$$

Thus:

$$(\text{m})^{\text{Control}} < (\text{m})^{\text{B/4AP}}$$

Notably B/4AP treatment caused an increased $\frac{\Delta[GSH]}{\Delta t}_{\text{BSO + BCNU}}$ in both H_2O_2 stressed and unstressed neurons (Fig 2.3C). This data confirms an increased GSH metabolism in electrically active neurons, which may mediate the sensitisation observed in Fig 2.1A. Furthermore, as observed in Figure 2.2

$$(\text{m} - (\text{r}))^{\text{Control}} > (\text{m} - (\text{r}))^{\text{B/4AP}}$$

despite the higher rate of (m) in B/4AP treated neurons, we can extrapolate that GSH recycling rate is also greater in electrically active neurons.

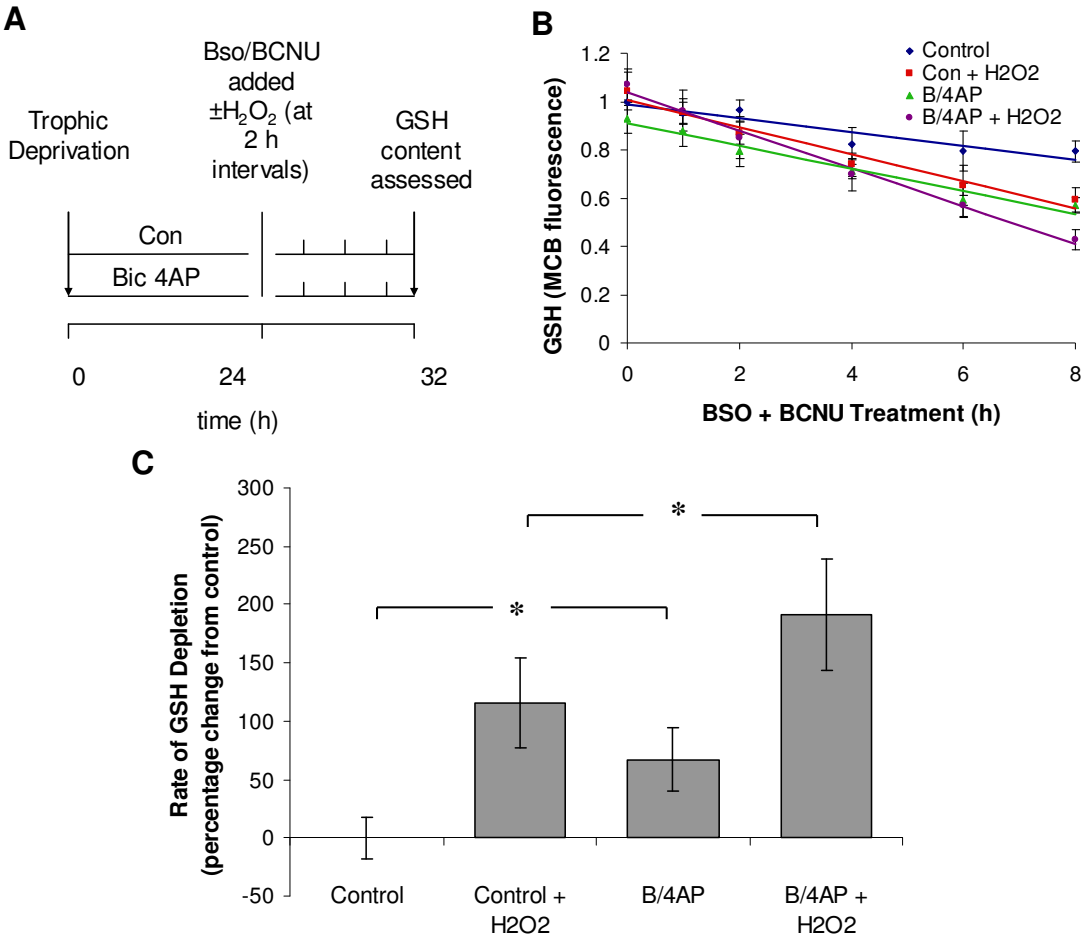

Figure 2.3. Synaptic activity increases metabolism of GSH. A) Schematic of experiment. After 24h in trophically deprived media, control and B/4AP treated neurons were stimulated with 100 µM BSO and 20 µM BCNU ± 100 µM H_2O_2 between 8 and 0 h, followed by MCB treatment and subsequent recording of fluorescence from cell lysates, normalised to protein content and respective control **B)** Graph showing average GSH levels at each time point and derived slope indicating rate of GSH depletion (n=7). **C)** Analysis of GSH depletion gradient of neurons treated with BSO and BCNU. Each experiment is normalised to their respective control, *p<0.05, n=7.

GSH depletion block is dependent on NMDAR but not on the presence of astrocytes

That synaptic activity would have an effect on GSH pathway enzymes was of great interest. We had previously shown that increased electrical activity upregulated genes regulating the antioxidant thioredoxin and peroxiredoxin pathways (Papadia et al., 2008), a process mediated by NMDAR signalling. Thus we investigated whether the maintenance of GSH levels observed in Fig 2.2 was dependent on the activation of NMDARs. Blocking NMDAR activation with MK801 abolished B/4AP mediated maintenance of GSH levels, increasing $\frac{\Delta[GSH]}{\Delta t}$ to that of control treated neurons (Fig 2.4A-B). Neurons treated with MK801 alone displayed a significantly increased $\frac{\Delta[GSH]}{\Delta t}$ compared to both control neurons and neurons treated with concurrently with B/4AP and MK801. The difference to controls is perhaps indicative of the increased vulnerability of neurons after NMDAR blockade (Ikonomidou et al., 1999; Ikonomidou et al., 2000); whilst the difference between MK801 treated neurons and B/4AP and MK801 treated neurons may be mediated by Ca^{2+} entry through voltage gated L-type Ca^{2+} channels, which may be opened in the presence of the neuron depolarising 4AP.

Astrocytes protect neurons from oxidative stress (Desagher et al., 1996) and astrocytic GSH synthesis has been shown to protect neurons from oxidative stress (Shih et al., 2003); GSH synthesis in known to be greater in astrocytes than neurons (Raps et al., 1989), and astrocytes are thought to supply neurons with GSH precursors (Dringen et al., 1999), indeed neurons co-cultured with astrocytes display greater GSH concentrations (Bolanos et al., 1996). Accordingly we tested whether the maintenance of GSH in B/4AP treated neurons was dependent on the presence of astrocytes by performing the experiment described in figure 2.2A on cultures treated with mitosis inhibitor AraC on Div 0, which reduces number of astrocytes in culture to below 0.1% (see methods). Maintenance of GSH levels of neurons in astrocytes free cultures treated with B/4AP was observed, matching the effect demonstrated in astrocytes containing cortical cultures (Fig 2.4C-D). This maintenance was lost in

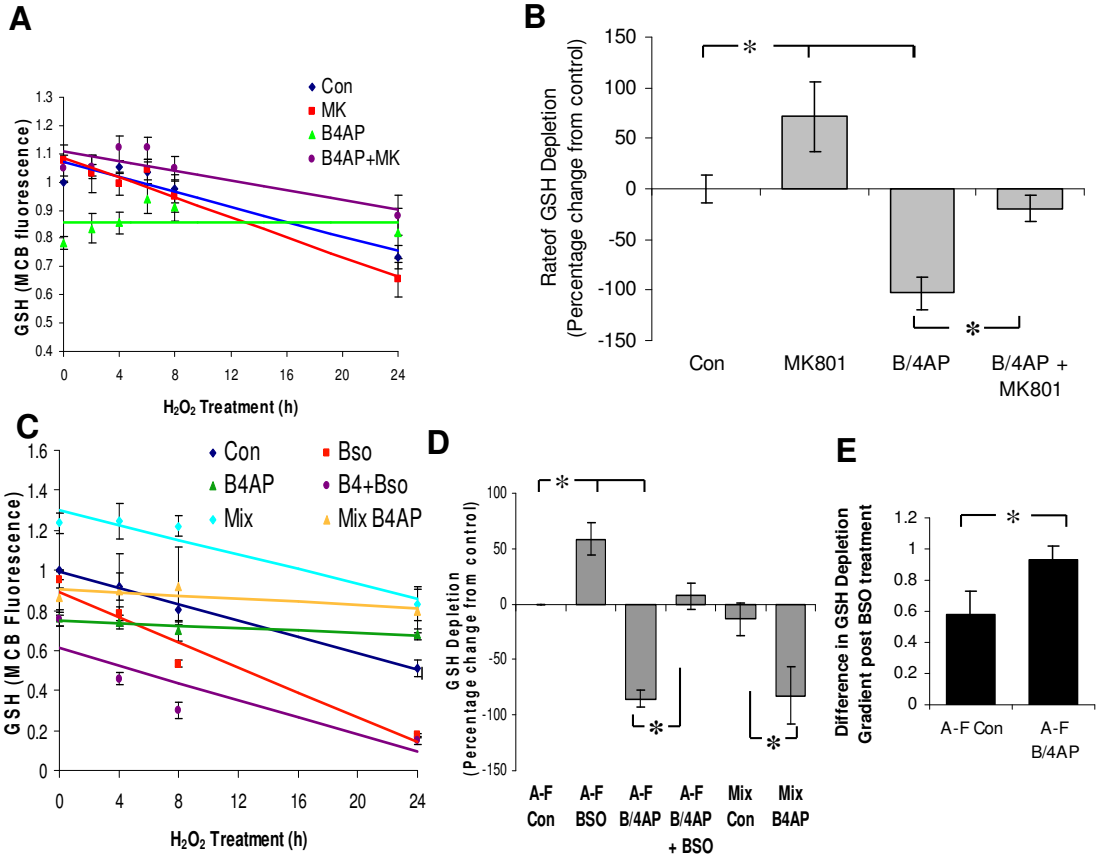

Figure 2.4. Block of GSH depletion is NMDAR dependent and Astrocyte independent. A,B) MK801 blocks B/4AP protection of GSH levels, neurons were treated as described by Fig 2.2A, except 50 μM MK801 was added 30 min before B/4AP to conditions indicated. **A)** Graph showing average GSH at each time point and derived slope indicating GSH depletion rate, and **B)** rate of GSH depletion normalised to control (n = 7, *p <0.05). **C,D)** B/4AP treatment protects GSH levels in the absence of astrocytes, neurons treated at Div 0 with AraC (A-F: Astrocyte Free) and neurons prepared normally (Mix – AraC treated at Div 5) were treated in parallel as described in Fig 2.2A. **C)** Graph showing average GSH content at each timepoint and slope indicating GSH depletion rate, and **D)** rate of GSH depletion normalised to Early AraC treated control (n = 4, *p < 0.05). **E)** Difference in GSH depletion of neurons from Astrocyte Free cultures between control and BSO treatments (n = 4, *p <0.05).

the presence of BSO, and the rate of GSH synthesis (as determined by the formula $(\frac{\Delta[GSH]}{\Delta t} - \frac{\Delta[GSH]}{\Delta t}_{BSO})$) was greater in B/4AP treated astrocytes-free neuronal cultures (Fig 2.4E), again matching observations demonstrated in astrocytes containing cultures. This indicates that the upregulation of GSH synthesis observed was intrinsic to neurons and independent of the presence of astrocytes in our cultures.

Synaptic activity upregulates GSH pathway enzyme activity

Whilst the previous experiments provide a novel cellular method of investigating GSH production, metabolism and recycling, we wished to confirm the proposed increase in GSH pathway enzyme activity through established enzymatic assays. Analysis of lysates of neurons would moreover be instructive as to the capacity of enzymatic activity; which in the case of GCL, though modulated by post-translational changes, is predominantly determined by the expression of its subunits (Dickinson and Forman, 2002; Franklin et al., 2009). Thus to confirm the activity of GSH pathway specific enzymes we performed canonical enzyme assays for each using cell lysates obtained from cortical cultures treated for 24 h with B/4AP and/or MK801. GCL activity was recorded using a fluorescent probe NDA, which forms covalent bonds to the GSH precursor glutamyl-cysteine (White et al., 2003). B/4AP treatment significantly upregulated GCL activity in an NMDAR dependent manner, evidenced by MK801 block (Fig 2.5A). GR activity was determined by recording NADPH loss over time in the presence of excess GSSG (Carlberg and Mannervik, 1985); we observed an increase after B/4AP treatment that was not blocked by MK801 (Fig 2.5B). GPX activity was determined by recording NADPH loss in the presence of excess GSH and GR (Flohe and Gunzler, 1984); again activity of this enzyme was upregulated by B/4AP in an NMDAR independent manner. Crucially these data confirm the increased GSH pathway enzyme activity in synaptically active neurons suggested in previous figures.

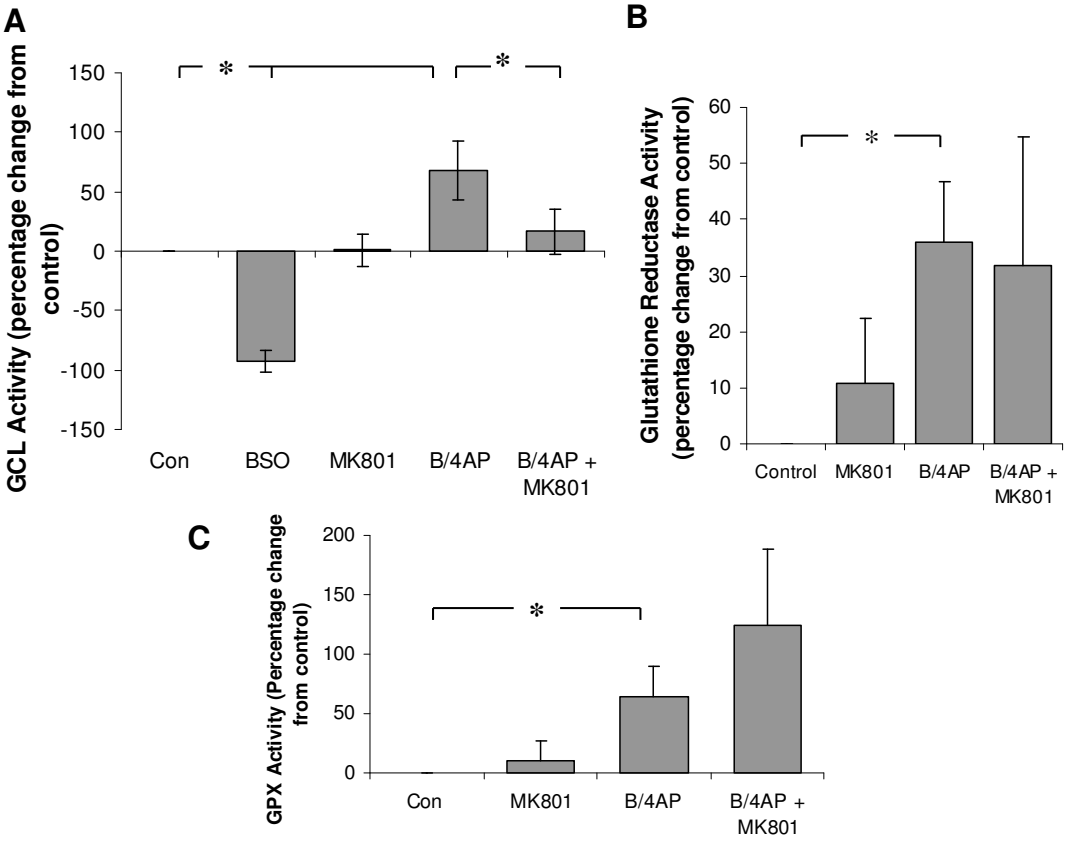

Figure 2.5. Synaptic activity increases GSH pathway enzyme activity. **A)** Glutmaylcysteine Ligase activity; neurons were treated for 24 h as indicated, with 50 μM MK801 added 30 min before, then lysates were analysed for GCL activity, normalised to protein levels, then to control activity (n = 6, *p <0.05). **B)** Glutathione Reductase activity; neurons were treated for 24 h as indicated, then lysates were analysed for GR activity, normalised to protein levels, then to control activity (n = 9 con and B/4AP, n = 6 MK801 and B/4AP + MK801, *p <0.05). **C)** Glutathione Peroxidase activity; neurons were treated for 24 h as indicated, then lysates were analysed for GPX activity, normalised to protein levels, then to control activity (n = 8, *p <0.05).

Synaptic activity upregulates GSH pathway enzyme mRNA expression

It has been established that synaptic activity can mediate neuroprotection through the regulation of particular gene expression (Zafra et al., 1992; Papadia et al., 2008; Zhang et al., 2009), moreover changes in GCL enzyme activity are predominantly influenced by changes in its subunit mRNA expression (Dickinson and Forman, 2002; Franklin et al., 2009). Accordingly we investigated whether the upregulation of GSH pathway enzyme activity was concurrent with an increase in their mRNA expression. Real-time qPCR was performed using cDNA produced from RNA isolated from neurons simulated with B/4AP and/or MK801 for 24 h. Of the subunits involved in GSH synthesis GCLC, encoding the catalytic subunit of GCL, was upregulated by synaptic activity in a NMDAR dependent manner (Fig 2.6A). GCLM, the modifying subunit of GCL, and GSS, encoding GSH synthetase the final but not rate-limiting step of GSH synthesis, were not upregulated by B/4AP treatment (Fig 2.6B). GSR, encoding the recycling enzyme GR, was upregulated by synaptic activity in an NMDAR independent manner (Fig 2.6C), mirroring the result of the enzymatic assay. As mentioned before, the GPX family consists of at present 8 known members; we investigated the expression of the selenocysteine containing GPXs 1-4, as GPX6 is known to be solely expressed in olfactory bulb. Of the genes investigated, GPX4 and GPX2 were both upregulated by B/4AP treatment, with regulated GPX4 in an NMDAR dependent manner (Fig 2.6D, E).

To establish a causal link between the upregulation of mRNA to the increased GSH pathway enzyme activity, we the GCL assay on lysates obtained from neurons treated with the transcription inhibitor actinomycin D. GCL activity was inhibited by actinomycin, blocking any increase conferred by B/4AP stimulation (Fig 2.7). Taken together we can conclude that synaptic activity increases GSH synthesis through the upregulation of mRNA.

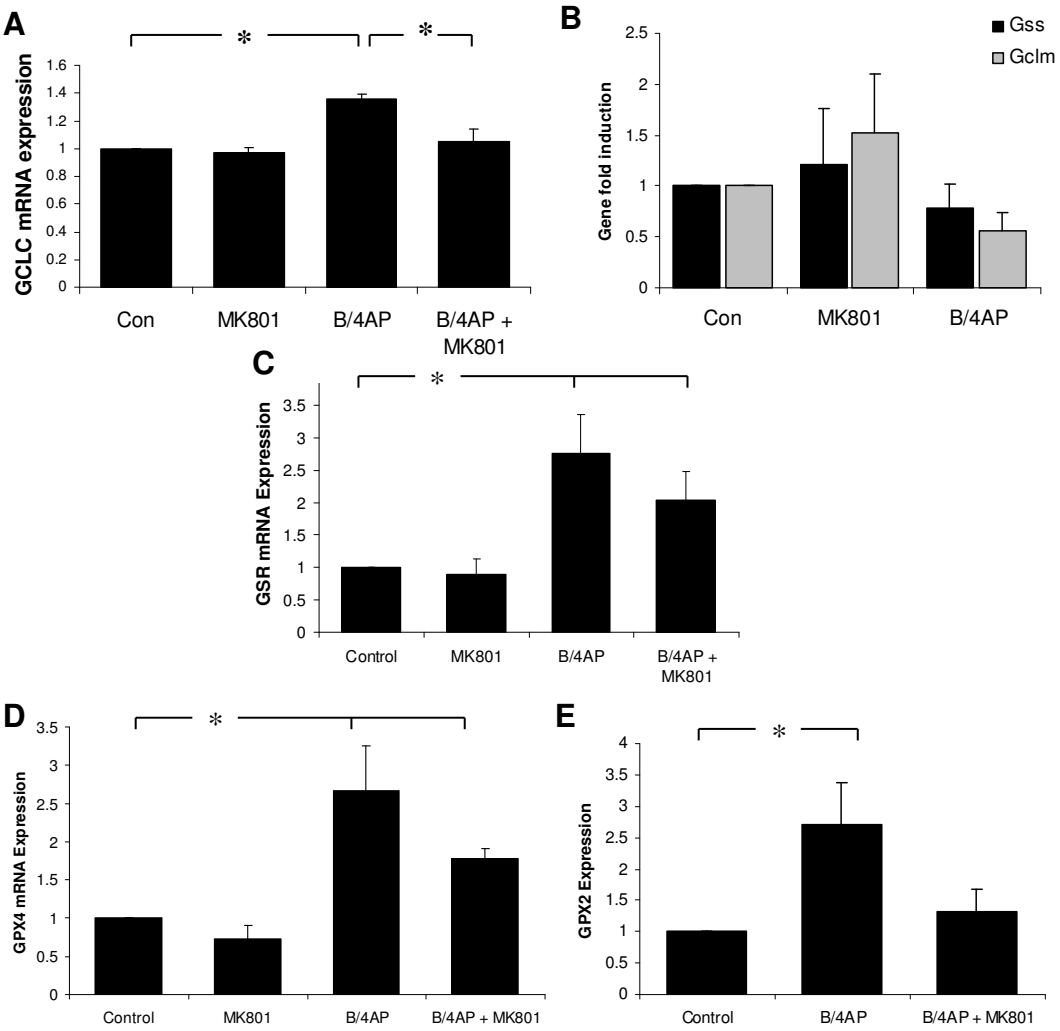

Figure 2.6. Synaptic activity upregulates GSH pathway enzyme mRNA expression. Neurons were treated as indicated for 24 h, mRNA was then isolated, converted to cDNA, and analysed using qPCR, expression normalised to respective control. **A)** γGC ligase catalytic subunit (GCLC) mRNA expression (n = 3, *p <0.05). **B)** γGC ligase modifier subunit (GCLM) and GSH synthetase (GSS) mRNA expression (n = 3). **C)** GSH Reductase (GSR) mRNA expression (n = 4, *p <0.05). **D)** GSH Peroxidase 4 (GPX4) mRNA expression (n = 4, *p <0.05). **E)** GSH Peroxidase 2 (GPX2) mRNA expression (n = 9, *p<0.05).

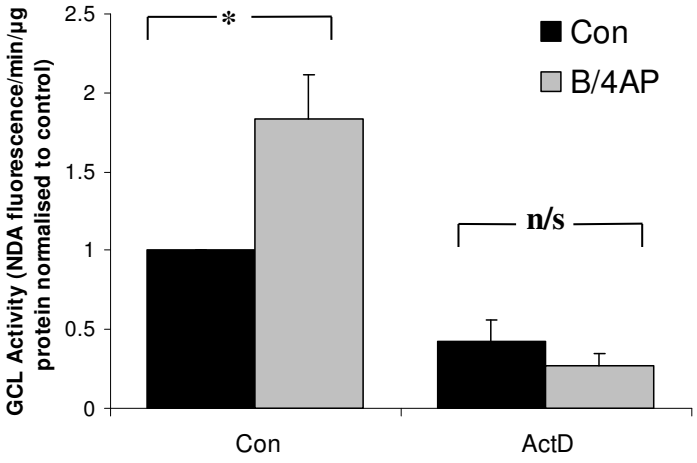

Figure 2.7. Inhibition of transcription blocks increase in GSH pathway enzyme activity. Neurons were treated for 24 h as indicated with 10 µM Actinomycin D added 30 min before B/4AP. Cells were then lysed and lysates were assayed for GCL activity, normalised to protein levels, then to control activity (n = 4, *p <0.05).

Upregulation of GR promoter activation by NMDAR signalling

To investigate the mechanism through which synaptic activity upregulates GSH pathway enzyme mRNA we employed the use of promoter-reporter plasmids. Negligible reporter activity and/or induction by B/4AP treatment was found from previously published GCLC (Yang et al., 2005) or GPX4 (Maiorino et al., 2003) constructs, suggesting that transcriptional regulation of these promoters occurs outwith these sites. Significant signal was observed however using mouse GSR promoters: a 'full promoter' (- 1253 to + 624) and a previously identified ARE site (- 857 to – 725) (Harvey et al., 2009). B/4AP treatment significantly increased full promoter activity, in an NMDAR dependent manner (Fig 2.8A) in contrast to the enzymatic and qPCR data. It has been shown that the ARE transcription site sequences may contain an AP-1 site (Nguyen et al., 2003), and synaptic activity may stimulate transcription of some ARE regulated genes through AP-1 signalling (Papadia et al., 2008; Soriano et al., 2009). To explore the possible role of AP-1 signalling in GSR upregulation we co-transfected neurons with either reporter constructs and TAM-67 (a dominant-negative form of c-Jun (Brown et al., 1993)) to disrupt AP-1 signalling. Compared to globin controls, TAM-67 inhibited B/4AP mediated induction of GSR-ARE reporter activity (Fig2.8B). However, TAM-67 was unable to block induction of the full promoter by synaptic activity (Fig 2.8C), indicating that inhibition of AP-1 signalling is not sufficient to disrupt GSR induction by B/4AP treatment. Thus the increase in GSR mRNA by synaptic activity may be caused by the recruitment of multiple transcription factor pathways.

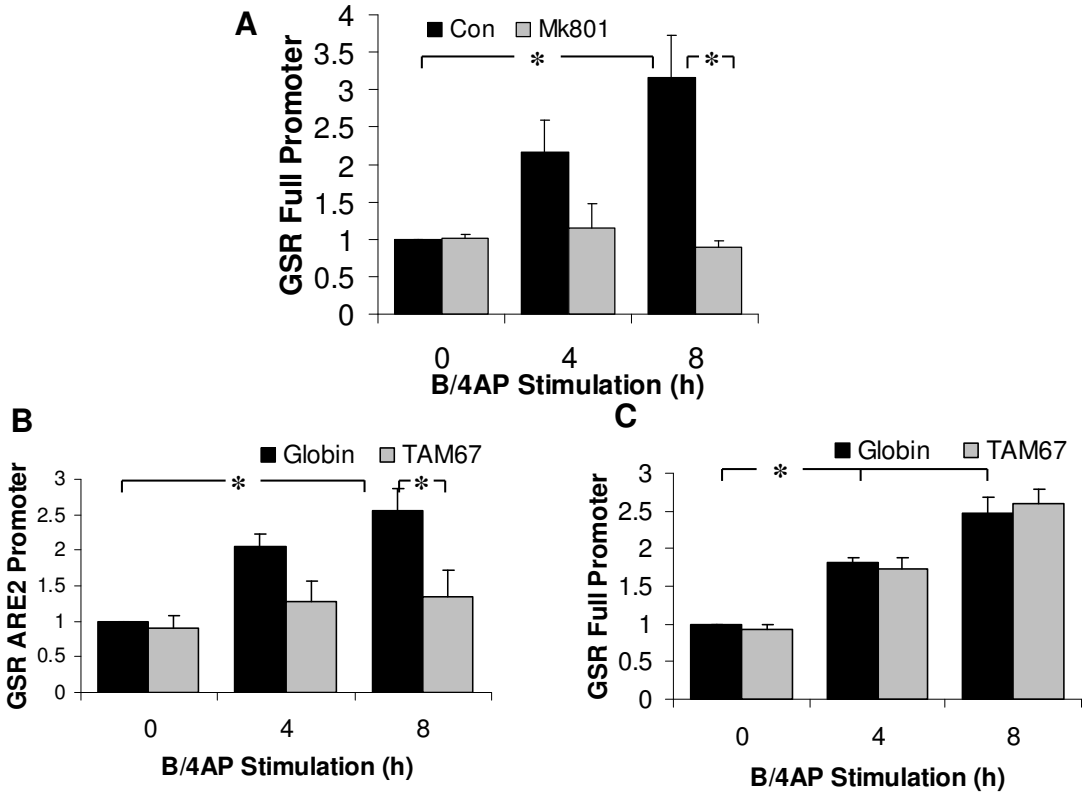

Figure 2.8. Glutathione Reductase promoter activity is increased by synaptic activity. A) GSR full promoter is activated by synaptic activity in an NMDAR dependent manner. Neurons were transfected with GSR full promoter and pTK Renilla. 24 h post transfection neurons were stimulated as indicated, with 50 μM MK801 added 30 min before, then Luciferase expression was measured and normalised to Renilla control (n = 4, *p < 0.05). **B)** AP-1 inhibition impairs B/4AP treatment activation of GSR-ARE(2). Neurons were transfected with GSR-ARE(2) reporter, pTK renilla, and either β-Globin or TAM67. Neurons were treated as indicated 24 h post-transfection, and Luciferase expression was recorded and normalised to Renilla control (n = 4, *p <0.05 to β-Globin B/4AP 8 h). **C)** AP-1 inhibition does not impair B/4AP treatment activation of GSR full promoter. Neurons were transfected with GSR full promoter, pTK Renilla and either β-Globin or TAM67 (n = 4, *p <0.05 to β-Globin control).

Blockade of NMDARs decreases GCL activity and expression *in vivo*

We wished to determine whether the effects of increasing synaptic activity on the GSH pathway were recapitulated *in vivo*. Although NMDAR blockade had little effect on enzyme expression or activity compared to controls *in vitro*, this may be explained by the minimal activation of these receptors in untreated conditions (Hardingham et al., 2001b), thus we hypothesised that MK801 treatment in juvenile rats would have a more pronounced effect, due to an increased NMDAR activation *in vivo*.

P7 rats were subjected to two consecutive intraperitoneal injections of either vehicle or MK801, at 0 and 8 h, and sacrificed 12 h after the initial treatment. MK801 treatment had no effect on total GSH levels (Fig 2.9A), GR activity (Fig 2.9B) or GPX activity (Fig 2.9C). However, GCL activity was strongly inhibited by NMDAR blockade (Fig 2.9D). Real-time qPCR analysis showed significant inhibition of GCLC, GSR and GPX4 mRNA expression in samples obtained from MK801 treated rats (Fig 2.9E). We decided to check if the loss of GCL activity would impact on GSH levels after a longer period of NMDAR blockade, and whether changes in GSR and GPX4 mRNA expression might manifest changes in enzyme activity. Accordingly we analysed rats similarly treated but sacrificed 24 h after first MK801 treatment. Whilst this had no significant effect on GR or GPX activity (data not shown), a reduction in GSH levels was observed (Fig 2.9F). These data suggest that similar to observations made *in vitro*, NMDAR activation is required for GSH pathway enzyme regulation *in vivo*, through control of their mRNA expression, modulation of which precedes changes in the activity of each enzyme.

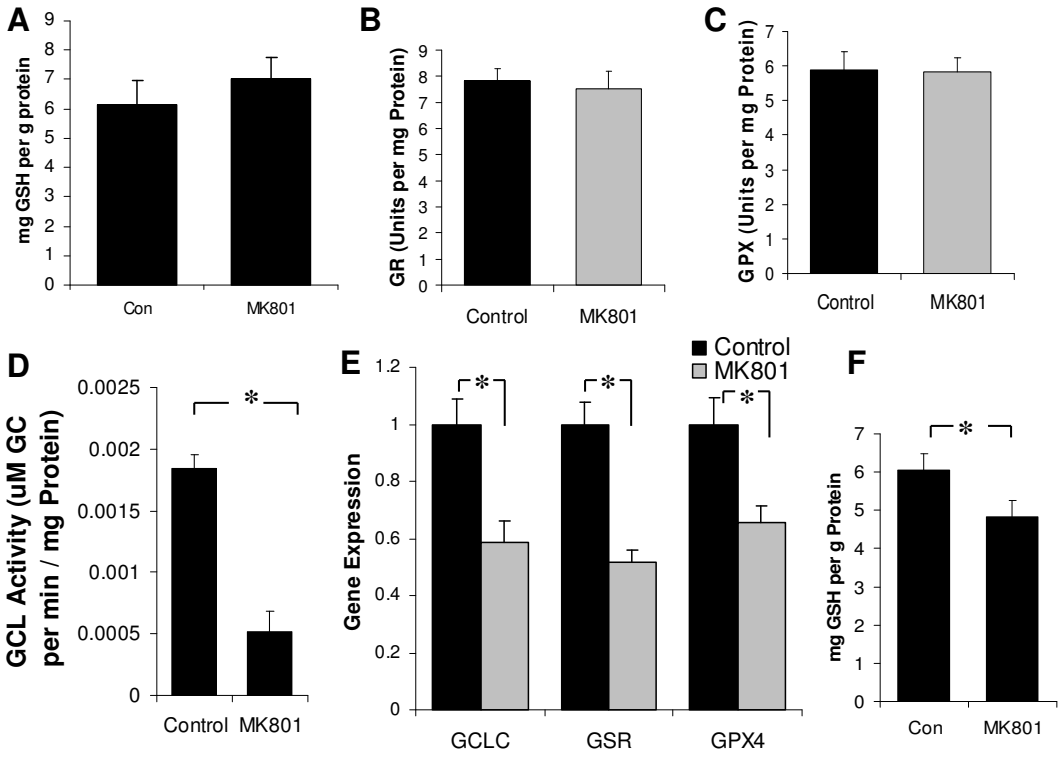

Figure 2.9. Blockade of NMDAR decreases GCL enzyme activity and GSH pathway enzyme mRNA *in vivo*. P7 rats received two consecutive intraperitoneal injections of saline vehicle (Control) or 0.5 mg/kg MK801 at 0 and 8 h. 12 h after first injection rats were sacrificed and frontal corticies were collected and snap-frozen in liquid nitrogen. **A,B,C and D)** GSH levels, GSH reductase and GSH peroxidase activities were unaffected by MK801 treatment, whereas γGC ligase activity was significantly reduced. Frontal corticies were lysed on ice and lysates assayed and normalised to protein levels. **A)** GSH colorimetric assay (n = 6 animals per condition). **B)** GSH Reductase activity assay (n = 6 animals per condition). **C)** GSH Peroxidase activity assay (n = 6 animals per condition). **D)** γGC ligase activity assay (n = 4 animals per condition, *p <0.01). **E)** GSH pathway enzyme mRNA expression was reduced by MK801 treatment. Corticies were lysed, mRNA isolated, converted to cDNA and expression of GCLC, GSR and GPX4 analysed by qPCR (n = 4 animals per condition, *p <0.01 to respective control). **F)** GSH levels detected from P7 rats treated as above, but sacrificed 24 h after first injection (n = 4 animals per condition, *p <0.05 one-way t-test)

Discussion

This study demonstrates that synaptic activity, through enhanced NMDAR signalling, coordinates changes in gene expression of enzymes of the GSH pathway, hitherto uncharacterised as activity-regulated genes. These changes in mRNA expression lead to increased enzymatic activity which mediates in part the neuroprotection conferred by synaptic activity against oxidative insult.

Synaptic activity promotes GPX activity

We have previously demonstrated that NMDAR stimulation strongly protects neurons against oxidative insult through upregulation of the peroxide neutralising thioredoxin-peroxiredoxin pathway (Papadia et al., 2008), thus the observation that synaptic activity *enhanced* cell death to sub-toxic doses of hydrogen peroxide in GSH depleted neurons was indeed surprising. This suggests that active neurons have an enhanced metabolic rate and production of ROS that requires neutralisation by the GSH pathway. Inhibition of *de novo* GSH synthesis and recycling demonstrated further that synaptic activity increased the rate of GSH loss which, whilst the increased activity of other GSH utilising enzymes such as GST cannot be excluded, is coordinated with an increased GPX activity and the upregulation of GPX2 and GPX4 mRNA.

The GPX family consists of 8 currently known isozymes, each considered to fulfil certain distinct tissue and cellular specific roles (Brigelius-Flohe, 1999). Whilst no change was observed in the expression of the ubiquitous cytosolic GPX1, the predominant GPX in all cells, gastrointestinal GPX2, its most similar isozyme in terms of substrate specificity (Chu et al., 1993), was upregulated at an mRNA level. Upregulation of GPX2 is intriguing; it is known to be dynamically regulated by oxidative stress through activation the antioxidant response element (ARE) (Singh et al., 2006); furthermore, studies evaluating GPX mRNA levels in cell lines treated with selenium (required for the selenocysteine active site) deficient media show a

preferential stability, and increased resynthesis after selenium repletion, of GPX2 over GPX1 (Brigelius-Flohe, 1999).

Phospholipid GPX4 is distinct from other peroxide neutralising enzymes in its broad substrate specificity, which crucially allows it to reduce lipid hydroperoxides (Ursini et al., 1982; Maiorino et al., 1990). Lipids, in particular polyunsaturated fatty acids, are particularly sensitive to oxidative stress. Abstraction of a single hydrogen atom from a lipid side chain by ROS produces a carbon-centred lipid radical, which through reaction with oxygen and neighbouring lipids sets off a free radical chain reaction, self propagating whilst producing lipid hydroperoxides (Halliwell, 1992). This severely disrupts membrane integrity, which is of course essential for cellular function. The importance of GPX4 is highlighted by the embryonic lethality of GPX4 knockout, as compared to the oxidative stress sensitising effects of GPX1 or GPX2 knockout (Brigelius-Flohe, 2006). Neurons, due to their high content of polyunsaturated fatty acids, are particularly vulnerable to lipid peroxidation (Coyle and Puttfarcken, 1993), thus GPX4 activity is essential. Within the CNS GPX4 is preferentially expressed in neurons (Savaskan et al., 2007), and its overexpression protects neurons from oxidative insult (Ran et al., 2006). Thus GPX4 upregulation presents an intriguing mechanism by which neurons protect themselves from the increased ROS production that is associated with increased synaptic activity, either through increased metabolic demand or activation of ROS producing secondary messenger systems (Brennan et al., 2009).

NMDAR activation promotes GCL activity in an astrocyte independent manner

Given the importance of *de novo* synthesis of GSH to neuronal function and viability; the regulation of GCLC by synaptic activity is an exciting prospect. Whilst the observed protection against GSH depletion after hydrogen peroxide insult could be mediated by the known increased activity of the thioredoxin-peroxiredoxin detoxifying pathway, this effect is concurrent with increased GCL activity and GCLC mRNA expression. Although B/4AP stimulation did not increase neuronal GSH content *per se*, this is in accordance with the feedback inhibition of GCL

activity by GSH (Richman and Meister, 1975); which is notably absent in the *in vitro* GCL enzyme activity assay used. This increased GCL activity could be due to post-translational modification of the enzyme. GCL activity can be decreased by phosphorylation of its catalytic subunit by Ca^{2+}/Calmodulin-dependent kinase II, protein kinase C and PKA (Sun et al., 1996) which are all present in neurons; thus theoretically synaptic activity regulated phosphatases, such as calcineurin, could increase GCL activity. However, the increased GCL activity was blocked by transcription inhibitor actinomycin, demonstrating the importance of *de novo* mRNA expression. Factors increasing GCLC mRNA expression have been shown to invariably lead to increased protein expression (Franklin et al., 2009), and this mechanism is considered to be the predominant method of increasing GSH synthesis (Dickinson and Forman, 2002).

The dependence of GCLC mRNA upregulation on NMDAR signalling is instructive, especially when taken in context with the loss of GSH level maintenance after NMDAR blockade. This suggests that the reduction of GSH depletion is NMDAR dependent, and due to an increased GSH biosynthesis. GR and GPX activity and mRNA upregulation were not abolished by NMDAR blockade *in vitro*, which could suggest that increased activity of these enzymes, GR in particular, is not sufficient to protect neuronal GSH levels. However *in vivo*, NMDAR blockade reduced mRNA expression of both of these enzymes, and study of the GR promoter showed an NMDAR dependent activation by synaptic activity. Depolarisation by 4AP allows the activation of postsynaptic L-type calcium channels, which can activate similar downstream pathways as those recruited by NMDAR dependent Ca^{2+} influx (Hardingham et al., 1999), this could potentially be sufficient to drive GR and GPX gene transcription despite NMDAR blockade. This suggests a possible difference in Ca^{2+} threshold required for GR/GPX transcriptional regulation that may be lower than that required for regulation of GCLC.

Astrocytes are known to have greater GSH content than neurons (Raps et al., 1989), and many studies investigating GSH bioavailability within the CNS have focused on the ability of astrocytes to provide GSH precursors to neurons (Dringen et al., 1999;

Shih et al., 2003). Whilst this is without doubt a crucial method of protecting neurons from oxidative stress, the data presented here demonstrates a way neurons can protect themselves independently of astrocytes, through NMDAR signalling. Importantly, this does not preclude the role astrocytes in influencing neuronal GSH levels. Selective neuronal knockdown of either GCL subunit by shRNA causes sensitisation to oxidative and nitrosative insult and neuronal death, which is not rescued by coculturing with astrocytes (Diaz-Hernandez et al., 2005). Thus neurons require GCL enzyme activity to facilitate astrocyte mediated protection against oxidative insult, to synthesise GSH from astrocytes supplied GSH; the synergistic effect of stimulating GSH synthesis in both neurons and astrocytes is explored in greater detail in chapter 5.

The NMDAR dependent increase in GCL activity is especially important given the vulnerability of neurons undergoing NMDAR blockade (Ikonomidou et al., 1999; Papadia et al., 2008). The data observed in juvenile rats injected with MK801 showed a decrease in overall GSH levels after 24 h; however mRNA of GSH pathway enzymes and GCL activity were already decreased at 12 h. This highlights the ability of GCL to maintain GSH levels for a limited time despite a reduced capacity, and moreover suggests that maintenance of the GSH pathway by NMDAR activity could be vital for neuronal viability and may contribute to the toxic effect of NMDAR blockade (Ikonomidou et al., 1999). Intriguingly, in adult mice MK801 treatment does not cause widespread apoptosis, but sensitises neurons to subsequent insult (Ikonomidou et al., 2000); analogously, whilst incomplete inhibition of GCL might not deplete GSH levels of otherwise unstressed neurons, GSH levels would likely be decreased by subsequent oxidative insult.

Regulation of GSH pathway by synaptic activity

Our previous study established a causal link between NMDAR activation and the downregulation of the thioredoxin inhibitor Txnip; the expression of which is elevated in ageing human brain and could influence vulnerability to oxidative stress (Papadia et al., 2008), potentially due to reduced synaptic activity. Intrinsic to the

oxidative stress theory of ageing (Harman, 1956) is the depletion of GSH in various organs of the body (Hazelton and Lang, 1980; Maher, 2005), which parallels a reduced dynamic regulation of GCL activity (Suh et al., 2004; Morrison et al., 2005); furthermore GSH depletion is observed in neurodegenerative disease, such as Parkinson's disease (Perry et al., 1982). An intriguing possibility is that NMDAR signalling, that is protective in mature CNS (Ikonomidou et al., 2000), and its regulation of the GSH pathway is perturbed in age-related diseases.

Exercise and environmental enrichment are both implicated with increased glutamatergic signalling (Molteni et al., 2002; Nichols et al., 2007) and reduction of cerebral oxidative stress (Herring et al., 2010); and are both implicated in neuroprotection (Young et al., 1999; Liebelt et al., 2010). Whether these are epiphenomena, or due to NMDAR regulation of GSH pathway enzymes is of great interest; study of this pathway could lead to therapeutic strategies to slow neurodegeneration in the variety of disorders associated with oxidative damage. Of particular importance, identification of the pathway that links NMDAR signalling to GSH pathway enzyme mRNA upregulation would be of great benefit to those attempting to generate such strategies.

Chapter 5
Neuroprotection by Nrf2 mediated activation of the glutathione antioxidant pathway

Introduction

GSH is selectively lost from neurons in a number of neurodegenerative diseases (Perry et al., 1982; Liu et al., 2004; Chi et al., 2007; Babu et al., 2008); strategies that increase GSH synthesis have shown some success in inhibiting ROS mediated damage and neuronal death in models of these disorders (Boyd-Kimball et al., 2005; Vargas et al., 2008; Chen et al., 2009). Having demonstrated an increase in GSH synthesis capacity regulated by NMDAR signalling we wished to investigate alternative methods of maintaining GSH levels in neurons. It is possible that NMDAR dependent signalling regulation of GSH is compromised by ageing or disease, as reports have independently observed perturbations of GSH levels and of NMDAR signalling (Greenamyre et al., 1987; Ulas et al., 1992; Liu et al., 2004). Thus alternative methods of increasing the expression of the GSH pathway may be able to rescue the activity of this antioxidant pathway in neurons.

Activation of the Nrf2 transcription factor, through inhibition of binding to its negative regulator Keap1, causes an upregulation of a battery of genes involved in the regulation of the GSH pathway (Vargas and Johnson, 2009). We resolved to investigate if Nrf2 activation could similarly maintain GSH levels in neurons, thus mimicking this aspect of NMDAR signalling protection. To stimulate Nrf2 dependent gene transcription we utilised the triterpenoid CDDO-F3, a potent activator of Nrf2 that has been shown to strongly promote transcription of Nrf2 regulated genes and confer neuroprotection (Pitha-Rowe et al., 2009; Stack et al., 2010; Neymotin et al., 2011). Whilst the Nrf2 pathway is known to be more active in astrocytes than neurons (Kraft et al., 2004; Chen et al., 2009), its activation in astrocytes is still sufficient to protect neurons (Vargas et al., 2008; Calkins et al., 2010). This protection is in part mediated by the release of GSH precursors from astrocytes (Shih et al., 2003; Diaz-Hernandez et al., 2005). We have previously demonstrated the neuroprotective effect of Nrf2 overexpression in neurons (Soriano et al., 2008b), and hypothesised that CDDO treatment might lead to GSH pathway enzyme upregulation in neurons. Moreover, since neuroprotection against oxidative insult by astrocytes is dependent on neuronal GCL activity (Diaz-Hernandez et al.,

2005), we postulated that CDDO treatment might stimulate GSH synthesis in both cell types in an additive manner.

We observed that CDDO-F3 treatment of cortical cultures promoted an Nrf2 dependent increase in astrocytic but not neuronal GSH levels. This increase was sufficient to protect neurons and maintain their GSH levels in response to oxidative insult in an astrocyte dependent manner; removal of astrocytes from our culture preparations abolished both the neuroprotection and GSH level maintenance of neurons treated with CDDO-F3. We established that CDDO-F3 treatment increased GCL activity in astrocytes, coinciding with an increased expression of both GCL subunit mRNA expression; however similar treatment of pure neuronal cultures bore no effect on GCL. To characterise this further we investigated the effect of CDDO-F3 treatment on cultures obtained from both Nrf2 knockout and Keap1 knockout mice. Genetic ablation of Nrf2 in astrocytes caused a large reduction in GCL subunit expression, whilst Keap1 knockout astrocytes displayed an increased constitutive expression of these subunits, consistent with the known regulation of GCLC and GCLM by Nrf2. However, knockout of either Nrf2 or Keap1 had no effect on GCL subunit expression of neurons, indicating its independence of Nrf2 mediated transcription. We demonstrated the requirement of GSH export from astrocytes by the transporter MRP1 for neuroprotection by CDDO-F3 treatment, and that the GSH precursor γGC was sufficient to protect neurons and rescue GSH depletion in the presence of GCL inhibitor BSO. Finally, we observed an additive protection of neurons and maintenance of GSH levels in electrically active neurons co-treated with CDDO-F3, demonstrating that increased GSH synthesis in both cell types is able to potently protect neurons from oxidative stress.

Results

CDDO-F3 upregulates GSH levels of astrocytes but not neurons in an Nrf2 dependent manner

We previously showed that activation of the Nrf2 pathway with the small molecule chemopreventative agent D3T prevented neuronal apoptosis and induced expression of sulfiredoxin (Soriano et al., 2008b). Since GCLC is a known Nrf2 target gene (Sekhar et al., 2002) we wished to see if activation of the Nrf2 pathway would lead to an increase in GSH content. Analysis of GSH levels of lysates harvested from rat cortical neuronal cultures after 24 h treatment with Nrf2 inducers D3T, CDDO-EA and CDDO-F3 showed no increase in GSH, whilst treatment with BSO or H_2O_2 significantly reduced GSH levels (Fig 3.1A). Pure astrocytic cultures treated with Nrf2 inducing drugs showed significant increases in GSH levels, including in response to H_2O_2 treatment, with the greatest increase caused by CDDO-F3 (Fig 3.1A). Nrf2 wildtype mice astrocyte cultures showed a smaller but significant increase in GSH content, which was not present in Nrf2 knockout astrocytes cultured and treated in parallel (Fig 3.1B), indicating the dependence of Nrf2 activation in the increase in GSH content. ImageJ analysis of MCB fluorescence intensity of rat neuronal cultures allowed separate determination of GSH levels in neurons and astrocytes of these 'mixed' cultures; whilst this revealed no increase of GSH content in neurons, a significant increase in astrocyte specific GSH after 24 h CDDO-F3 treatment was observed (Fig 3.1C), confirmed by post-hoc GFAP immunocytochemistry (Fig 3.1D). H_2O_2 treatment also caused an increase in astrocytic GSH content. The increase of GSH in astrocytes by CDDO-F3 seemed substantially less in mixed cultures compared to pure astrocytic cultures, but was nonetheless significant; however since astrocytes make up around 5% of our rat cortical neuronal cultures (see methods, and (Soriano et al., 2008b)), this increase is insufficient to be recorded in examination of lysates harvested from mixed neuronal cultures. Thus these data suggest that Nrf2 activation by CDDO treatment promotes the synthesis of GSH in our cortical cultures, but that this is restricted to the astrocytes.

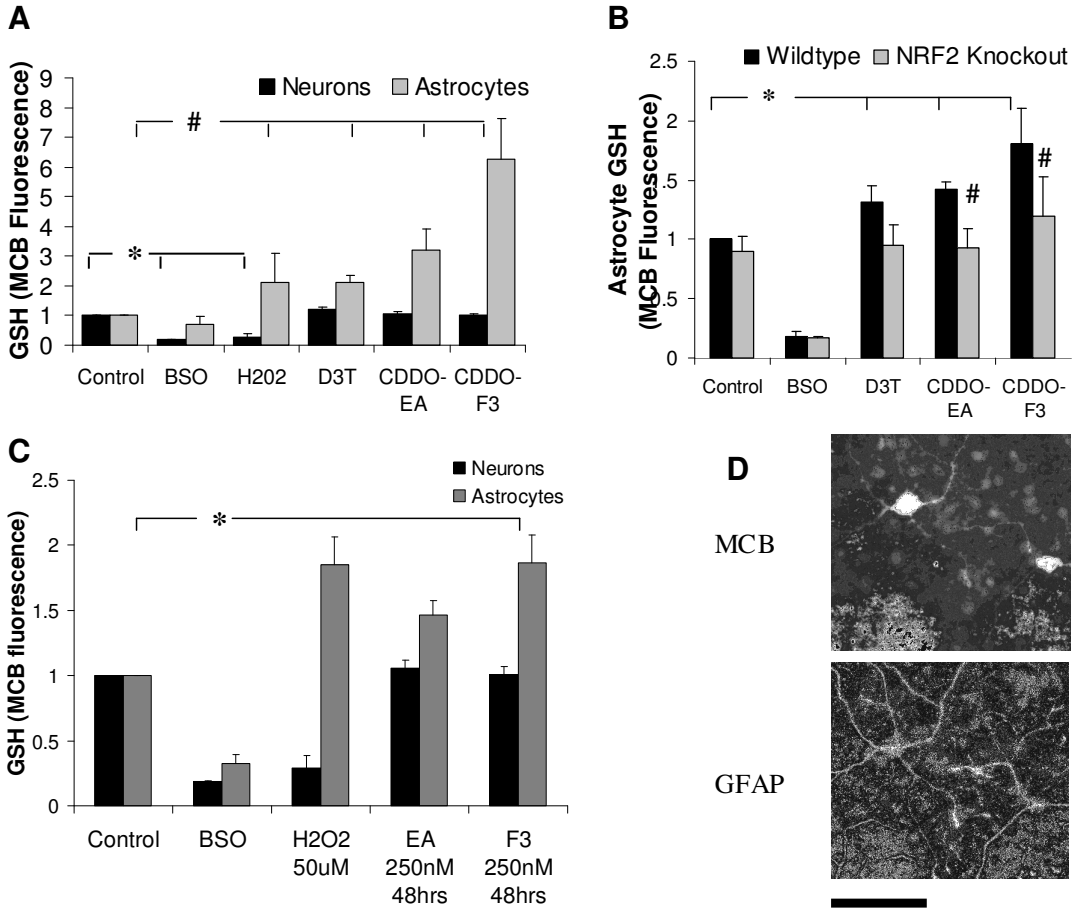

Figure 3.1. Synthetic triterpenoid CDDO-F3 increases astrocytic but not neuronal GSH levels. **A)** Neuronal and pure astrocytic rat cultures were treated for 24 h in trophically deprived medium with 100 µM BSO, 25 µM D3T, 250 nM CDDO-EA and 250 nM CDDO-F3 (throughout unless otherwise indicated). 30 min prior to termination of stimulation cells were treated with 50 µM MCB. Cells were then lysed and analysed for MCB fluorescence, and normalised to protein content and respective control (n = 7, *p <0.05 to neuron control, #p <0.05 to astrocyte control). **B)** Pure astrocytic cultures were prepared from Nrf2 $^{-/-}$ mice and treated for 24 h as indicated. MCB fluorescence was recorded from lysates and normalised to protein content and respective control (n = 6, *p <0.05 to control, #p <0.05 to wildtype). **C,D)** Neuronal rat cultures were treated as indicated for 24 h. After 30 min MCB treatment cells were washed with warm fresh TMo and pictures of MCB fluorescence were taken. Nuclear fluorescence intensity was recorded using ImageJ, and normalised to respective control. To confirm astrocytic identity post-hoc GFAP immunocytochemistry was performed (example pictures shown in **(D)**, scale bar = 20 µM). (n = 3, *p <0.05)

CDDO-F3 protects neurons from oxidative damage in an astrocyte and Nrf2 dependent manner

We have previously observed that small molecule inducers of Nrf2, and neuronal Nrf2 overexpression could confer potent neuroprotection against oxidative stress (Soriano et al., 2008b); whilst astrocytic overexpression of Nrf2 has also been demonstrated to protect neurons through an increased synthesis of GSH (Shih et al., 2003). Whilst changes in GSH levels by CDDO treatment were segregated to the astrocytes in unstressed cultures, we wished to determine the relevance of this to neuroprotection. Rat cortical mixed neuronal cultures treated with a single dose of CDDO-F3 250 nM 24 h before insult were protected against H_2O_2 stimulation (Fig 3.2A). However in astrocytes free neuronal cultures, this protection was abolished (Fig 3.2A). To determine the Nrf2 dependence of this protection we repeated these experiments in Nrf2 wildtype and knockout mice. CDDO-F3 protection against oxidative insult by treatment with two doses of 100 nM CDDO-F3 was lost in cortical mixed neuronal cultures prepared from Nrf2 knockout mice (Fig 3.2B). Thus neuroprotection by CDDO treatment is both Nrf2 and astrocyte dependent.

CDDO-F3 inhibits GSH depletion dependant on the presence of astrocytes

Whilst CDDO stimulation did not raise GSH levels in unstressed neurons, we had previously observed an increase in GSH synthesis capacity of electrically active neurons that did not result in increased GSH levels in unstressed conditions. This lack of increase was presumably due to feedback inhibition of GCL by GSH. Whilst CDDO treatment required the presence of astrocytes to protect neurons, it is known that the Nrf2 transcriptional pathway is stronger in astrocytes than neurons (Kraft et al., 2004). Nrf2 mediated transcription from the ARE upregulates a battery of genes related to the GSH pathway including both GCL subunits (Vargas and Johnson, 2009). We hypothesised that upregulation of enzymes of the GSH pathway induced by CDDO treatment might still occur in neurons, but that this effect was masked by feedback inhibition; thus we decided to investigate the effect of CDDO-F3 treatment

on $\frac{\Delta GSH}{\Delta t}$ in response to H_2O_2 stimulation (as described in Fig 2.2). 24 h preconditioning of rat cortical cultures with CDDO-F3 caused a significant decrease in $\frac{\Delta GSH}{\Delta t}$ compared to controls (Fig 3.3A-B). This maintenance of GSH levels was abolished when H_2O_2 stimulation was concurrent with GCL inhibitor BSO, indicating an increased GSH synthesis in cells. In contrast to observations made from electrically active neurons there was no difference in $\frac{\Delta GSH}{\Delta t}_{+\ \mathrm{BSO}}$ between control and CDDO-F3 treated cells, suggesting no increase in GR activity by CDDO treatment. To determine the role of astrocytes in the effect of CDDO-F3 treatment on $\frac{\Delta GSH}{\Delta t}$, these experiments were performed in parallel with regular neuronal cultures (with astrocytes making ~ 5% of total cell number) and astrocyte free neuronal cultures. The maintenance of GSH levels in neurons treated with CDDO-F3 was not observed in astrocytes free cultures (Fig 3.3C-D). These data suggest that the effects of CDDO treatment on the GSH antioxidant pathway are restricted to astrocytes, though this is sufficient to both maintain neuronal GSH levels and protect neurons from oxidative insult.

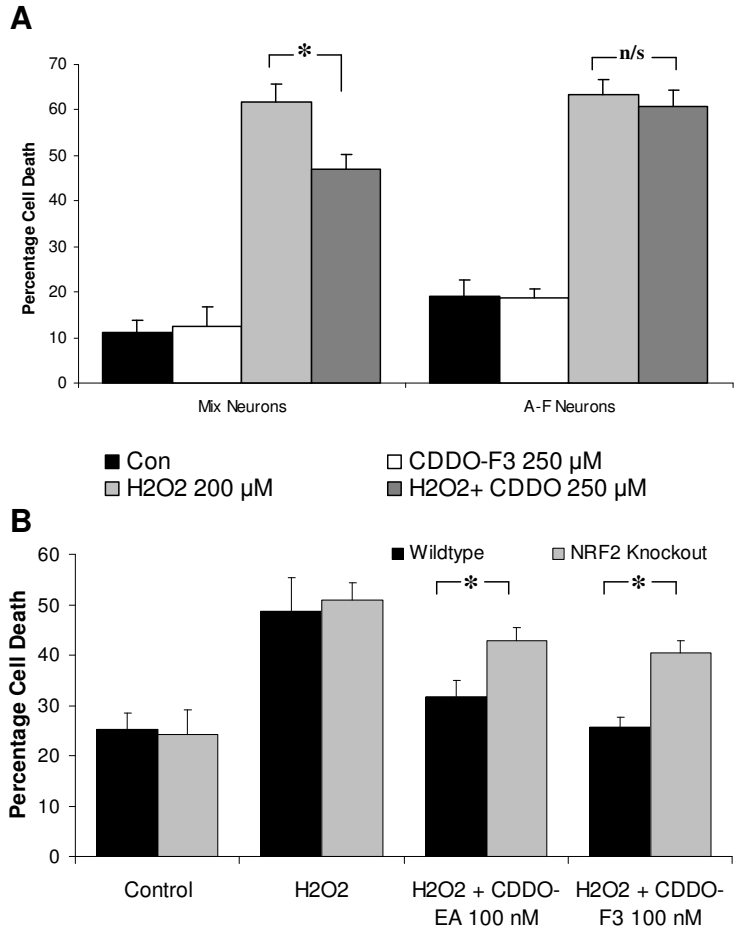

Figure 3.2. CDDO-F3 protects neurons from oxidative damage in an astrocyte and NRF2 dependent manner. A) Rat cortical neuronal cultures (Mix Neurons, AraC Div 5) and pure neuronal cultures (A-F Neurons, Astrocyte Free - AraC Div 0) were treated with CDDO-F3 for 24 h in TMo, then with 200 µM H_2O_2 overnight. Cells were then fixed, nuclei stained with DAPI, and cell death was measured by counting pyknotic and non-pyknotic nuclei (n = 4, *p <0.05 to H_2O_2). **B)** Neuronal cultures were prepared from Nrf2 $^{-/-}$ mice and treated with two consecutive doses of 100 nM CDDO at 0 and 23 h. At 24 h cells were treated with 100 µM H_2O_2 and left overnight. Cells were then fixed and DAPI stained and cell death recorded (n = 5, *p <0.05 to wildtype).

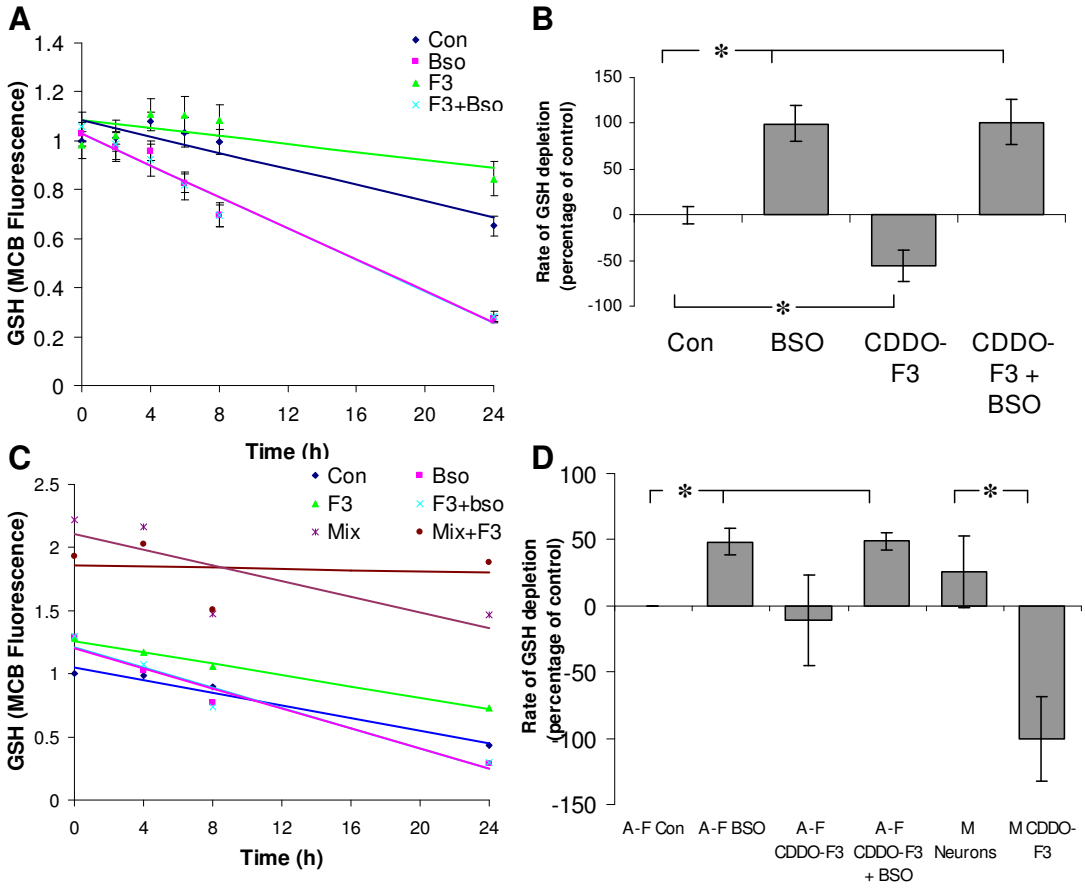

Figure 3.3. CDDO-F3 inhibits GSH depletion in an astrocyte dependent manner. A,B) CDDO-F3 treatment protects GSH levels. Rat neuronal cultures were treated as described in Fig 2.3A, with CDDO-F3 for 24 h and 100 µM BSO added 30 min before H_2O_2 treatment where indicated. **A)** shows average GSH levels at each timepoint and curve indicating GSH depletion rate. **B)** GSH depletion rates, normalised to control (n = 6, *p <0.05). **C,D)** CDDO-F3 treatment does promote the maintenance of GSH levels in the absence of astrocytes. Neuronal cultures (M - Mix Neurons, AraC Div 5) and pure neuronal cultures (A-F – Astrocyte Free, AraC Div 0) were treated as above. **C)** shows average GSH levels at each timepoint and curve indicating GSH depletion rate. **D)** GSH depletion rates, normalised to control (n = 5, *p <0.05).

CDDO-F3 upregulates GCL enzyme activity and GCLC expression in astrocytes

To confirm the effect of Nrf2 activation by CDDO-F3 on GSH synthesis we performed a cell-free GCL enzymatic assay on lysates obtained from mixed neuronal cultures treated with CDDO-F3 for 24 h. A two-fold increase of GCL activity was observed (Fig 3.4). Running these samples in parallel with those obtained from similarly treated astrocytes free cultures and pure astrocytic cultures revealed striking differences in GCL activity between these preparations; when normalised to protein content, astrocytes displayed a greater basal GCL activity, in accordance with the known increased presence of the enzyme in astrocytes (Raps et al., 1989). Furthermore, the induction of enzyme activity was greater in astrocytic cultures, whilst CDDO-F3 treatment of astrocytes free neuronal cultures failed to increase GCL activity (Fig 3.4).

Since changes in GCL enzymatic activity are largely governed by the expression of its two subunits (Franklin et al., 2009), we investigated mRNA expression of GCLC and GCLM using qPCR. The results observed closely agreed with those made in the GCL enzymatic assay; basal mRNA expression in astrocytes was increased compared to mixed neuronal cultures and astrocyte free cultures, whilst CDDO-F3 treatment caused an upregulation of both GCLC (Fig 3.5A) and GCLM mRNA expression (Fig 3.5B) in pure astrocytic but not astrocyte free cultures.

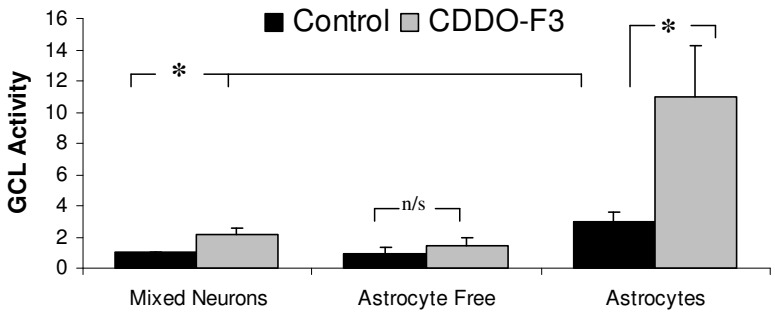

Figure 3.4. CDDO-F3 increases GCL activity in astrocytes not neurons. Neuronal (Mixed Neurons, AraC Div 5), pure neuronal (Astrocyte Free, AraC Div 0) and pure astrocytic cultures were treated in parallel with CDDO-F3 for 24h. Cells were then lysed and assayed for GCL activity; activity normalised to protein level and neuronal culture control rate (n = 5, *p <0.05 to control)

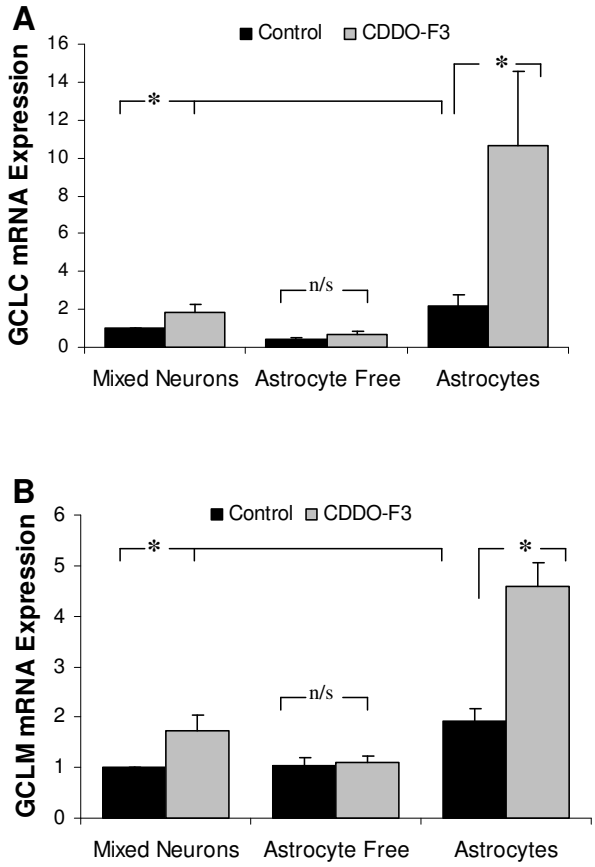

Figure 3.5. CDDO-F3 increases GCLC and GCLM mRNA expression in astrocytes not neurons. Neuronal (Mixed Neurons, AraC Div 5), pure neuronal (Astrocyte Free, AraC Div 0) and pure astrocytic cultures were treated in parallel with CDDO-F3 for 24h. Cells were then lysed, mRNA was isolated, converted to cDNA and assayed for gene expression using qPCR. **A)** GCLC mRNA expression (n = 4, *p <0.05). **B)** GCLC mRNA expression (n = 4, *p <0.05)

Nrf2 and Keap1 regulate GCLC and GCLM in astrocytes but not neurons

The lack of induction of GCL enzymatic activity or subunit mRNA expression by CDDO-F3 treatment in neurons led us to hypothesise that this was due to a diminished role of Nrf2 signalling in these cells. To investigate this we analysed GCL subunit expression of cultures prepared from Nrf2 null mice. In pure astrocytic cultures, basal GCLC mRNA expression was significantly reduced in Nrf2 knockout cultures compared to wildtypes, with a complete loss of induction by CDDO-F3 (Fig 3.6A). In contrast, pure neuronal samples displayed no induction in GCLC or GCLM mRNA expression by CDDO-F3 treatment; and intriguingly, no difference in basal mRNA levels between Nrf2 wildtypes and knockouts (Fig 3.7A).

To examine this apparent Nrf2 independence further we analysed GCL subunit expression of cultures prepared from Keap1 knockout mice. These mice show constitutive induction of Nrf2 target genes (Wakabayashi et al., 2003), and in retinal pigment epithelial cells have increased levels of GSH (Gao and Talalay, 2004). Keap1 knockout astrocytes showed increased basal expression of GCLC, which occluded further upregulation by CDDO-F3 (Fig 3.7A). Basal GCLM expression was also elevated; though additional induction by CDDO-F3 treatment was observed (Fig 3.7B) indicating some Keap1 independence of either treatment or gene regulation. However, in pure neuronal cultures neither CDDO-F3 treatment nor Keap1 knockout had any effect on GCLC (Fig 3.7C) or GCLM mRNA expression (Fig 3.7D). Thus Nrf2 signalling, upon which basal GCLC mRNA expression and inducible increases in astrocytes are dependent, does not regulate GCL subunit mRNA expression in neurons.

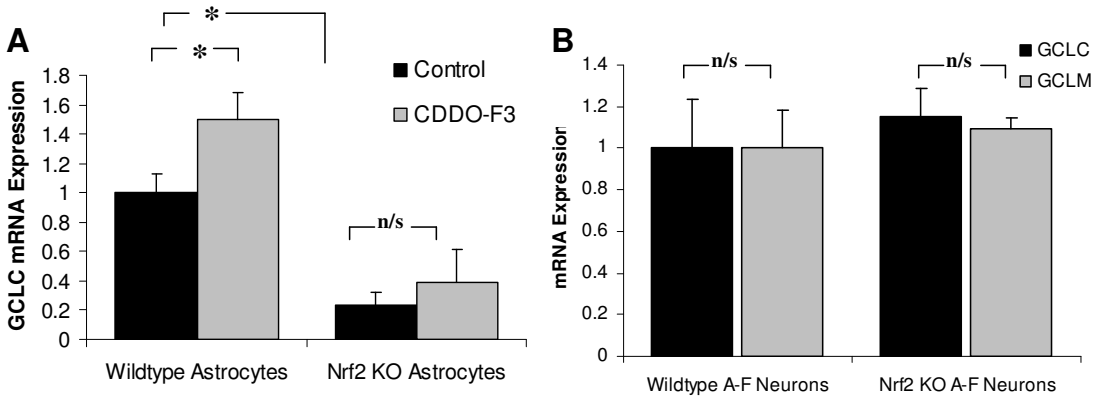

Figure 3.6. GCL subunit expression is regulated by Nrf2 in astrocytes but not neurons. A) Pure astrocytic cultures were prepared from Nrf2 $^{-/-}$ mice and treated with CDDO-F3 for 24 h, after which cells were lysed, mRNA isolated and GCLC mRNA analysed by qPCR (n = 8, *p <0.05 to control). **B)** Astrocyte free neuronal cultures (A-F Neurons, AraC Div 0) from Nrf2 -/- mice were stimulated for 24 h with CDDO-F3 and analysed for GCLC and GCLM expression (n = 4).

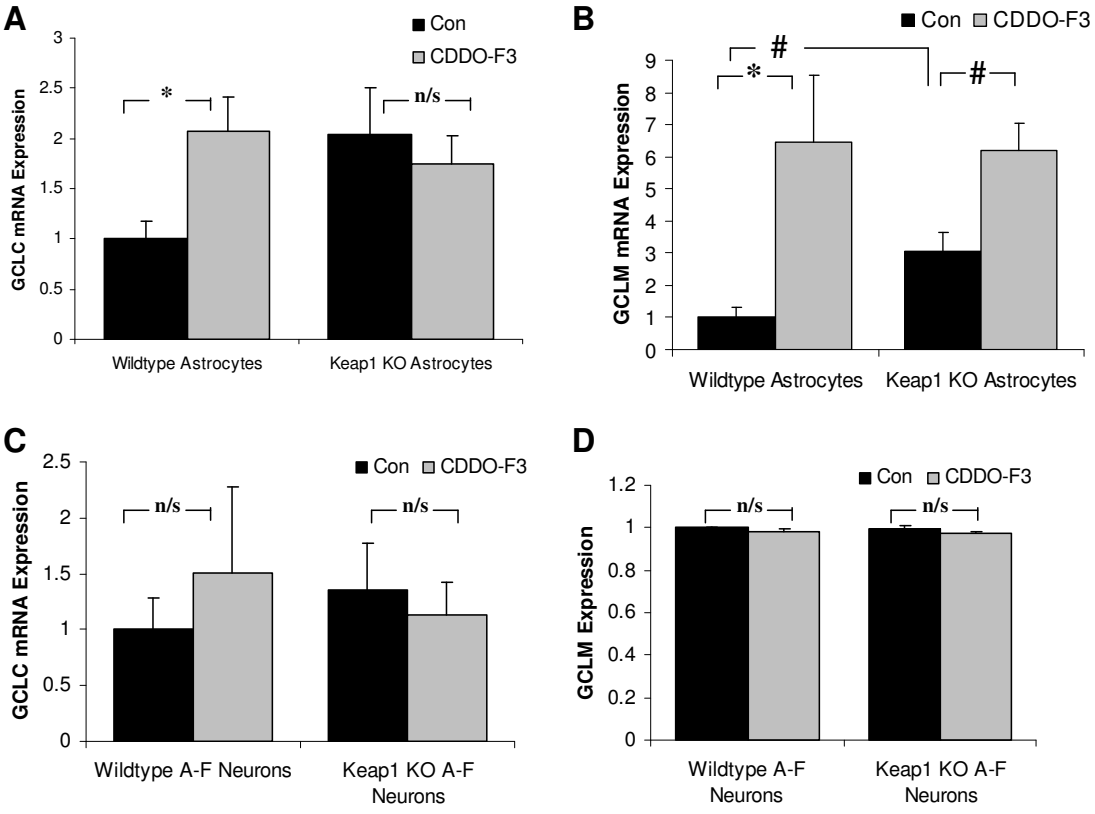

Figure 3.7. Keap1 regulates astrocytic GCL subunit mRNA expression but not neuronal levels. Cultures were prepared from Keap1 -/- mice and treated with CDDO-F3 for 24 h, after which cells were lysed, mRNA isolated and mRNA analysed by qPCR. **A)** GCLC mRNA expression from pure astrocyte cultures (n = 5). **B)** GCLM mRNA expression from pure astrocyte cultures (n = 4, *p <0.05 to wildtype control, #p <0.05 to Keap1KO control). **C)** GCLC mRNA expression from pure neuronal (A-F Astrocyte Free, AraC Div 0) cultures (n = 6). **D)** GCLM mRNA expression from astrocyte free neuronal cultures (n = 6).

GSH precursor γGluCys protects neurons from oxidative insult

Despite acting independently of neurons, Nrf2-mediated increase in GCL activity restricted to astrocytes was sufficient to protect neurons from oxidative insult and maintain GSH levels. Nrf2-overexpressing astrocytes have been shown to be neuroprotective (Shih et al., 2003), through the release of GSH. Release of GSH from astrocytes is known to be mediated in part by the transporter MRP1 (Cole and Deeley, 2006; Minich et al., 2006). To investigate the role of GSH release in mediating CDDO-F3 neuroprotection we used the potent MRP1 blocker MK571 (Leier et al., 1996), which inhibits the release of GSH from astrocytes (Minich et al., 2006). MK571 inhibited CDDO-F3 mediated protection against H_2O_2 (Fig 3.8A). Since neurons cannot import GSH (Vargas and Johnson, 2009) it is postulated that the positive effect on neuronal GSH levels by its astrocytic release are through two mechanisms: through non-enzymatic extracellular detoxification of ROS (Drukarch et al., 1998); or through breakdown by astrocytic extracellular membrane bound γglutamyltranspeptidase to CysGly (Dringen et al., 1999) and subsequent hydrolysis by aminopeptidase N to readily imported cysteine and glycine (Dringen et al., 2001). To explore this we examined the effect of GSH and its precursors CysGly and γGluCys on neuronal survival against oxidative insult (the effect of cysteine supplementation was not investigated due to its highly excitotoxic effect at high doses (Olney et al., 1990)). 500 μM γGluCys, but neither GSH nor CysGly, protected neurons against H_2O_2 administration (Fig 3.8B). Detection of GSH content using MCB showed that γGluCys, but not GSH or CysGly, protected neuronal GSH levels in the presence of GCL inhibitor BSO (Fig 3.8C), suggesting its whole incorporation into cells could allow neurons to bypass this step of GSH synthesis. To investigate if GSH synthesis in astrocytes or neurons led to an increased cysteine uptake, radioactive ^{35}S cysteine uptake assays were performed. Neither CDDO-F3 nor B/4AP treatment changed cysteine uptake (Fig 3.8D); indicating that increased GSH synthesis does not require an increase in cysteine uptake capacity.

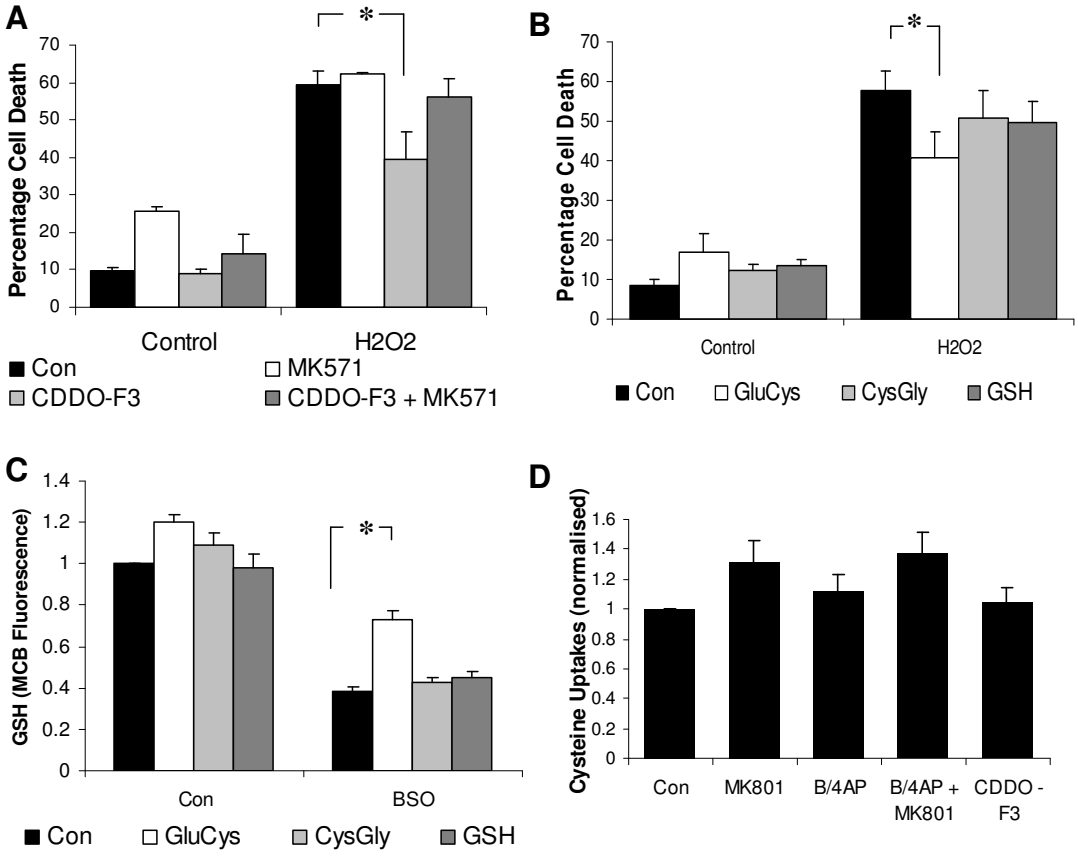

Figure 3.8. GSH precursor γGluCys protects neurons from oxidative insult. A) MRP1 inhibition blocks CDDO-F3 neuroprotection. Rat cortical cultures were treated with CDDO-F3 +/- 10 μM MK571, added 30 min before. After 24h cells were treated with 200 μM H_2O_2 overnight. Cells were then fixed, DAPI stained and cell death assesed by counting pyknotic and non-pyknotic nuclei (n = 4, *p <0.05). **B)** γGlutamylcysteine protects neurons against oxidative insult. Rat cortical cultures were treated with 500 μM of γglutamylcysteine, cysteineglycine or GSH. After 1 h, 200 μM H_2O_2 was added and cells were left overnight; then fixed, DAPI stained and assessed for cell death (n = 4, *p <0.05). **C)** γGlutamylcysteine sustains GSH levels in the presence of GCL inhibitor. Neurons were treated with 500 μM GluCys, CysGly and GSH; after 1 h cells were treated with 100 μM BSO for 24 h. After 24 h cells were treated with 50 μM MCB for 30 min, and lysates were analysed for fluorescence, with levels normalised to protein and controls (n = 4, *p <0.05 to BSO control). **D)** Cysteine uptake is not affected by modulation of synaptic activity or CDDO-F3 treatment. Neurons were treated for 24 h as indicated and ^{35}S cysteine uptake was analysed. Cysteine uptake was normalised to control rates (n = 4); cell numbers and death of neurons cultured and treated in parallel was also recorded as a control, with no significant differences observed (not shown).

CDDO-F3 and B/4AP treatment protect neurons in an additive manner

Having previously demonstrated an astrocyte independent upregulation of GSH synthesis by increased synaptic activity, and determined that the increase in GSH synthesis by Nrf2 induction is restricted to astrocytes, we wished to see if these two protective treatments would have synergistic effects. Stimulation with both CDDO-F3 and B/4AP protected neurons against H_2O_2 insult significantly more than either treatment alone (Fig 3.9A). We next investigated the effect of dual stimulation on GSH depletion. Since both B/4AP and CDDO-F3 treatment alone were sufficient to largely prevent the depletion of GSH by 100 μM H_2O_2, higher doses of H_2O_2 were used. Even after 24 h exposure to 250 μM H_2O_2, GSH levels in neurons treated with both CDDO-F3 and B/4AP were largely unaffected, whilst the Δ[GSH] of either treatment alone were significantly larger (Fig 3.9B). Thus upregulation of GSH synthesis in these two distinct cellular types, through two distinct molecular pathways, can be stimulated to have an additive effect on GSH levels, potently protecting neurons against oxidative insult.

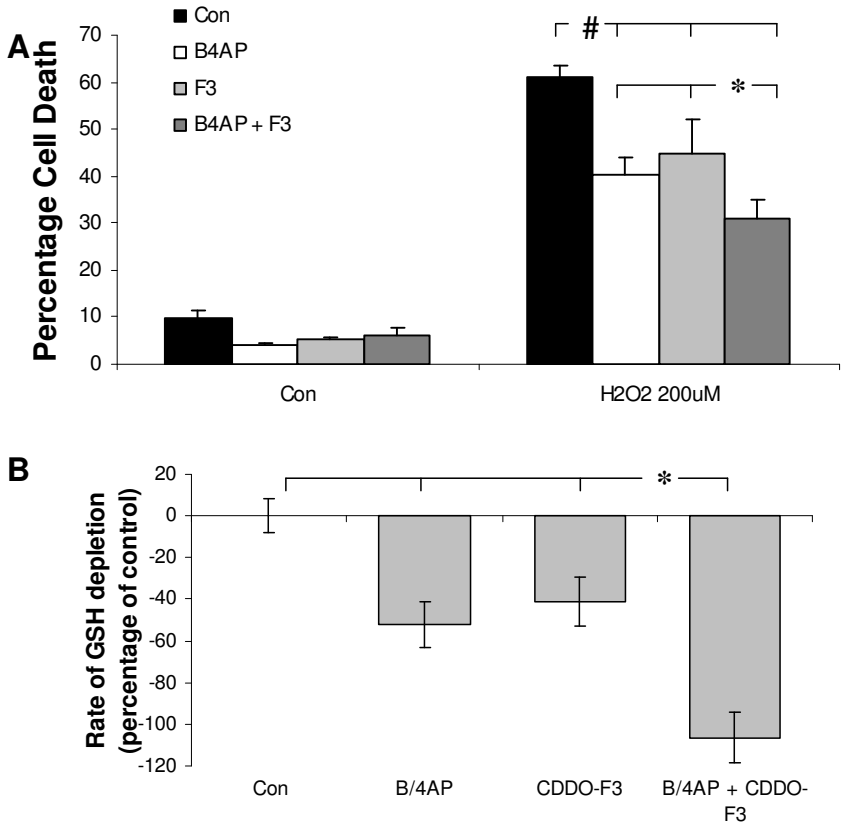

Figure 3.9. CDDO-F3 and B/4AP treatment protect neurons in an additive manner. A) Synaptic activity and Nrf2 activation potently protect against oxidative insult. Rat cortical cultures were treated with 50 μM bicuculline and 250 μM 4-aminopyridine (B/4AP) and CDDO-F3 either separately or simultaneously for 24 h. 200 μM H_2O_2 was then added overnight, cells were then fixed, DAPI stained and cell death calculated by counting number of pyknotic and non-pyknotic nuclei (n = 8, *p <0.05 to H_2O_2 treated B/4AP + F3, #p <0.05 to H_2O_2 control). **B)** Synaptic activity act synergistically to protect GSH levels. Neurons were treated as described in Fig 2.3A except H_2O_2 dose was increased to 250 μM; after 24 h simultaneous or separate treatment with B/4AP and CDDO-F3. MCB fluorescence was recorded from lysates, and normalised to protein content. GSH depletion over time was then calculated and rates normalised to control (n = 8 B/4AP, n = 4 CDDO-F3, *p <0.05 to B/4AP+F3).

Discussion

This study demonstrates the neuroprotective potential of the synthetic triterpenoid CDDO-F3, through Nrf2-dependent upregulation of GSH synthesis. Whilst Nrf2-dependent GSH regulation is restricted to astrocytes, the resultant upregulation is however sufficient to confer neuroprotection, dependent on the release of GSH. Separate upregulation of GSH synthesis in neurons and astrocytes by synaptic activity and Nrf2 signalling do not occlude each other, and can act additively to promote powerful neuroprotection.

GCL subunit mRNA expression is not regulated by Nrf2 in neurons

Both GCLC and GCLM are canonical Nrf2 regulated genes (Mulcahy et al., 1997; Solis et al., 2002), their basal expression is controlled by Nrf2 (Wild et al., 1999) and the attenuation of Nrf2 signalling causes a reduction in GSH synthesis in liver (Suh et al., 2004). Thus the finding that neuronal GCL subunit mRNA expression is unchanged between wildtype and Nrf2 knockouts is surprising. Whilst it is possible that compensatory genetic mechanisms, for instance possible regulation by Nrf1, could account for this lack of change; the results found in neurons are in stark contrast with those in astrocytes, which display reduced subunit expression in Nrf2 knockouts, and constitutive upregulation in Keap1 knockouts. The upregulation of Nrf2 target genes, particularly those involved in oxidant detoxification, by Keap1 knockout has been shown to be more extensive than that by CDDO (Yates et al., 2009). Thus the observation that neither genetic nor pharmacological activation of Nrf2 increased GCL subunit mRNA expression suggests firmly that Nrf2 dependent transcription does not regulate GSH synthesis in cortical neurons.

It has been demonstrated that the Nrf2 signalling pathway is diminished in neurons in comparison to astrocytes (Kraft et al., 2004; Chen et al., 2009), and that GSH synthesis in response to oxidative/nitrosative stress and Nrf2 inducers is restricted to astrocytes (Eftekharpour et al., 2000; Gegg et al., 2003). We have considered the further possibility that the Nrf2 transcriptional pathway is not at all active in neurons,

with current investigations in our lab indicating to that effect. H_2O_2 or Nrf2 activating compounds do not increase the expression of other Nrf2 target genes in neurons; neither does genetic ablation of Keap1. This is likely due to decreased expression of Nrf2 and/or increased expression of Keap1 in neurons. Loss of Nrf2 dependent transcription could be due to epigenetic changes, for instance histone deacetylation, which might restrict access to ARE transcription sites in neurons; however the Nrf2 target-gene sulfiredoxin can still be upregulated by neuron-specific Nrf2 overexpression (Soriano et al., 2008b), suggesting that the lack of Nrf2 dependent gene transcription may be due to diminished Nrf2 expression. However epigenetic changes of Nrf2 mRNA expression may underlie the loss of its transcriptional pathway in neurons. Strategies that induce an increase in Nrf2 mRNA expression may be able to rescue its signalling in neurons. Cyclo(His-Pro), an endogenous dipeptide produced by the cleavage of hypothalamic thyrotropin releasing hormone, has been demonstrated to increase Nrf2 mRNA expression in neurons, with a resultant increase in GSH synthesis and haemoxygenase-1 expression (Minelli et al., 2009). Thus it is probable that cyclo(His-Pro) may be able to rescue Nrf2 signalling, and concurrent treatment with CDDO-F3 could be able to potently increase GSH levels in neurons; this remains an intriguing future experiment.

CDDO-F3 is potently neuroprotective

Despite its effects on the GSH pathway being restricted to astrocytes, CDDO-F3 treatment was sufficient to protect neurons from oxidative insult, allowing them to maintain their GSH levels. This effect is remarkable considering that astrocytes represent only a small percentage of cells within our cultures. Nrf2 overexpression in astrocytes has been shown to confer profound neuroprotection (Shih et al., 2003; Chen et al., 2009; Calkins et al., 2010), predominantly through increased GSH synthesis and export. Our results agree, as MRP1 inhibition attenuated CDDO-F3 mediated protection; however there is some discrepancy with previous reports of GSH precursors. CysGly supplementation was ineffective at protecting neurons from subsequent oxidative insult, whereas γGluCys was protective. Since there is little evidence of a specific neuronal transporter for γGluCys, its ethyl-ester

conjugate which passes independently through membranes has been used to enhance neuronal GSH levels in models of compromised GCL activity *in vivo* (Drake et al., 2002), and has promising therapeutic potential as it can cross the blood brain barrier. However γGluCys itself has been shown to be transported into kidney cells (Anderson and Meister, 1983) and elevate neuronal GSH levels *in vitro* (Dringen et al., 1997b; Diaz-Hernandez et al., 2005; Le et al., 2010). We observed a rescue by γGluCys of GSH levels of neurons treated with GCL inhibitor BSO. Since equimolar GSH did not rescue GSH levels or protect against oxidative insult, we assume that this rescue is caused by γGluCys entry into cells and subsequent conversion to GSH by GSS, rather than through extracellular non-enzymatic scavenging of ROS that might alleviate stress on the GSH pathway.

Whilst we do not rule out the supply of cysteine to neurons by release and subsequent extracellular hydrolysis of astrocytic GSH, it is possible that CDDO-F3 treated astrocytes could mediate neuroprotection through supply of γGluCys. A potential mechanism for this, first proposed to explain the high concentration of γGluCys in urine of animals and humans with γglutamyl transpeptidase deficiency (Griffith and Meister, 1980), could be through cleavage of glycine from GSH by carboxypeptidase. Carboxypeptidases are essential in the CNS for cleaving C-terminal amino acids from neuropeptides (Fricker, 1988), and are excreted by astrocytes and neurons depolarised by KCl treatment (Vilijn et al., 1989). It is suggestive that carboxypeptidase E (the predominant neuronal isozyme) knockout mice show profound neurodegeneration, whilst its overexpression protects neurons against oxidative insult (Woronowicz et al., 2008). This could be an intriguing potential mechanism of astrocyte mediated neuroprotection that certainly requires further study

The unexpected nature of this result, and indeed all the GSH measurements made using MCB presented in chapters 4 and 5, signifies the importance of repeating these experiments using alternative techniques. Whilst MCB allows us to detect small changes in cellular GSH whilst using a minimum of material, two aspects of its function are a cause for concern: it may form fluorescent adducts with all thiol

containing compounds, albeit at a vastly reduced rate than the specific reaction to GSH catalysed by GST (Sun et al., 2006); there is also the potential that differences in GST expression in neurons could belie differences in MCB fluorescence, rather than actual GSH concentration. The incubation time of 30 minutes was specifically chosen to circumvent these problems, allowing the GST-catalysed exponential reaction of GSH with MCB to reach its plateau, whilst giving insufficient time to allow interference by non-specific thiol reaction to the remaining probe; however, it is imperative that we also use alternative GSH assay to recapitulate some these data. Future experiments shall also include alternative methods of inhibiting GCL activity, since the action of the GCL inhibitor BSO is not wholly specific, causing inhibition of the glutamyl amino acid transporter (Griffith et al., 1979) which may perturb other unkown biochemical pathways. To counter this, we shall use siRNA to knock down GCLC expression, and investigate the effect of this on B/4AP mediated neuroprotection.

CDDO-F3 and B/4AP treatment have an additive neuroprotective effect

Astrocytic synthesis of GSH is greater than that of neurons (Raps et al., 1989), with a portion of this GSH used to supply and protect neurons. Neurons co-cultured with astrocytes display higher GSH levels (Bolanos et al., 1996), a trend we also observed (Fig 3.3D). However, we have demonstrated that increasing synaptic activity by B/4AP stimulation confers a GSH synthesis dependent protection against oxidative insult that is independent of the presence of astrocytes (chapter 4). Since Nrf2 signalling to GCL subunit mRNA expression is restricted to astrocytes, we can conclude that the two GCL activating treatments used act through two distinct pathways, one Nrf2 dependent the other Nrf2 independent. As a proof of principle: B/4AP treatment upregulated GCL activity through increased GCLC mRNA expression, without increasing GCLM mRNA expression or GSH above unstressed/basal levels; whereas CDDO-F3 treatment increased (astrocytic) GCLC *and* GCLM mRNA expression, GCL activity *and* astrocytic GSH levels. GCLM alleviates the feedback inhibition of GCL by GSH (Huang et al., 1993), thereby facilitating extensive GSH synthesis. Thus whilst B/4AP treated neurons increase

GCL activity but not overall GSH levels, Nrf2 signalling in astrocytes is able to overcome feedback inhibition and increase GSH even in unstressed conditions through the upregulation of GCLM.

Whilst increasing synaptic activity alone had a profound neuroprotective effect against oxidative insult, concurrent activation of Nrf2 signalling in astrocytes had a further protective effect. Synaptic activity increased expression and activity of GSH pathway enzymes; thus stimulation with both treatments causes an interesting dynamic, whereby neurons are primed for GSH synthesis and metabolism, with astrocytes acting as providers of GSH. Circumstantial evidence for this additive protection is available from studies providing environmental enrichment, a conditioning known to increase glutamatergic signalling in animal models (Nichols et al., 2007), simultaneously with antioxidant-rich diet, which together strongly attenuate neurodegeneration in animal models (Pop et al., 2010).

Whilst bolus antioxidant supplementation has had mixed results as a therapeutic strategy, these results demonstrated here display an attractive therapeutic potential for Nrf2 activating compounds, which could be used to facilitate neuronal antioxidant defences; in particular the GSH pathway, which has shown to be compromised in neurodegenerative disease. Design and synthesis of Nrf2 activating compounds that can cross the blood brain barrier and target astrocytes may be a promising avenue of research, especially when used in concert with strategies that increase NMDAR regulated neuronal GSH synthesis.

Chapter 6
Concluding statement

Neuronal activity dependent protection against apoptotic and oxidative insults

Whilst the pathways, and even the cell type, studied during the course of this research have changed; the primary focus, study of signalling pathways that promote the protection of neurons, has not. I have presented here three different neuroprotective pathways:

- CREB-activating PACAP signalling, that mediates its protection not through activation of PKA alone, but by increasing the action-potential firing activity of the neuronal network.
- Synaptic-activity mediated neuronal increase of enzymes of the antioxidant GSH pathway.
- Nrf2 dependent increase of GSH synthesis, that although completely segregated to astrocytes, is sufficient to protect neurons from oxidative stress.

Each chapter has alluded to the protective potential of NMDAR signalling. In the first we demonstrated a previously unobserved requirement for on-going synaptic activity to mediate protection by the neurotrophic signalling molecule PACAP. In the second we revealed the influence of NMDAR signalling on the antioxidant GSH pathway. This observation was investigated further in the third chapter, where we demonstrated the Nrf2-independence of GSH synthesis in neurons, but through its activation in astrocytes we could promote a neuroprotective response, increasingly potent when concurrent with increased synaptic NMDAR signalling.

Our understanding of the protective potential of NMDAR signalling has advanced dramatically since the failure of antagonists as potential neuroprotective drugs for use in stroke and neurodegenerative disorders. Current therapeutic strategies based on this knowledge focus on the selective inhibition of extrasynaptic NMDAR activation. Memantine, an NMDAR antagonist that preferentially inhibits extrasynaptic NMDARs, has been shown to be well tolerated in clinical trials of cognitively impaired adults (Jones et al., 2007; Ott et al., 2007; Zhao et al., 2010), in stark contrast to the increased morbidity/mortality observed with classical NMDAR

antagonists (Ikonomidou and Turski, 2002). Recent observations of the multi-faceted protective potential of memantine in models of Huntington's disease (Okamoto et al., 2009; Milnerwood et al., 2010), suggest that therapies based around this strategy may be capable of, if not arresting, attenuating ongoing neurodegeneration caused by diseases of the CNS.

On the basis of the research presented here, a further strategy of accentuating the neuroprotective signalling by synaptic NMDARs, and of astrocytes, should be considered. This is particularly relevant when considering that ageing, the predominant risk factor of all neurodegenerative disorders, is associated with a decline in protective signalling. Endogenous Nrf2 pathway signalling has been observed to be attenuated in aged rodents (Suh et al., 2004; Duan et al., 2009); however this can be rescued by Nrf2 inducing agents, demonstrating their therapeutic potential. The neuroprotective potential of compounds such as CDDO-F3, as demonstrated in Chapter 5, has led to the proposal of their use as possible therapies for neuronal injury and neurodegenerative disorders (Vargas et al., 2008; Siebert et al., 2009; Alfieri et al., 2011; Neymotin et al., 2011). Alternatively naturally occurring compounds, such as polyphenols found in apple juice, have been shown to activate transcription of Nrf2 target genes (Soyalan et al., 2011), thus dietary regulation may have therapeutic potential in the treatment of neurodegenerative disorders (Ramesh et al., 2009; Remington et al., 2010).

Evidence that synaptic NMDAR signalling might be compromised with age is more circumstantial, though remains an intriguing possibility. Synaptic plasticity is attenuated with age in humans (Freitas et al., 2011), and decreased synaptic plasticity is a hallmark of many neurodegenerative diseases; in particular Alzheimer's disease, where a reduction of glutamatergic signalling is observed (Greenamyre et al., 1987; Ulas et al., 1992; Esteban et al., 2003). Furthermore soluble Aβ, a pathological hallmark of the disease, has been shown to impair neurotransmission and increase extrasynaptic NMDAR activation (Costa et al., 2009; Li et al., 2011). Exercise and environmental enrichment, factors demonstrated to potentiate glutamatergic signalling and synaptic plasticity (Molteni et al., 2002; Nichols et al., 2007), have

been shown to attenuate age-related and Alzheimer's disease neurodegeneration (Wilson et al., 2002; O'Callaghan et al., 2009; Lazarov et al., 2010). A recent study investigating the therapeutic potential of glucagon like peptide in Alzheimer's disease demonstrates this link between synaptic NMDAR signalling and neurodegenerative processes that occur in this disease: treatment reduced β amyloid plaques and soluble Aβ, whilst facilitating synaptic plasticity in both wildtype and disease model mice (McClean et al., 2011).

Possible pharmacological enhancement of synaptic NMDAR signalling is fraught with difficulty, due to the epileptogenesis associated with disinhibition of the CNS. However mild disinhibition by serotonin uptake inhibitor fluoxetine has recently been observed to restore synaptic plasticity in adult mice (Chen et al., 2011), a treatment which could in turn provide neuroprotection through enhanced synaptic NMDAR signalling. Another intriguing possible therapy could be the use of PACAP. As a neurohormone it can cross the blood brain barrier, and as observed in Chapter 3, it exerts its neuroprotective effects through enhancement of synaptic activity. PACAP has recently been shown to slow down Alzheimer's disease progression in mouse models (Rat et al., 2011), and is known to confer protection in a variety of neurodegenerative models (Reglodi et al., 2002; Reglodi et al., 2006; Tamas et al., 2006a; Vaudry et al., 2009).

Understanding of the intrinsic protective pathways of the CNS will eventually give rise to molecular strategies to combat the inevitable morbidity and mortality of neurodegenerative diseases and brain injury. A powerful tool in understanding these processes has been generated in the form of induced pluripotent stem cells (iPSCs). The generation of these cells (Takahashi et al., 2007) and their neuralisation (Chambers et al., 2009) represent the greatest scientific breakthrough of this century so far. Whilst cell replacement therapies, or complete substitution of rodent based models may be hyperbole at present, these cells do provide a valuable resource to augment our knowledge of neuroprotective signalling. In particular patient specific iPSCs will hopefully rapidly advance our understanding of neurodegenerative pathological processes. It remains an intriguing possibility that some of the findings

presented in this thesis may soon be recapitulated at a cellular level in human neurons.

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